ENDOGENOUS METABOLISM

OF

ARTHROBACTER GLOBIFORMIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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ABBREVIATIONS USED IN TEXT

ADP	Adenosine diphosphate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
a_w	Water availability
D	Dilution rate
DNA	Deoxyribonucleic acid
eqn.	Equation
NADH	Reduced nicotinamide adenine dinucleotide
NCIB	National Collection of Industrial Bacteria
p	Probability
PCA	Perchloric acid
pers. comm.	Personal communication
PHB	Poly-β-hydroxybutyric acid
p. no.	Page number
PYE	Peptone yeast-extract
$Q\,[O_2]$	Oxygen uptake, $\mu l\ (mg\ cells)^{-1}\ h^{-1}$
RNA	Ribonucleic acid
R.Q.	Respiratory quotient
R.T.	Room temperature
s.d.	Standard deviation
s.e.	Standard error

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SUMMARY

Endogenous metabolism is important in microbial survival. Microorganisms from the supposed starvation conditions of soil have a low rate of endogenous metabolism in contrast to those with a plentiful energy supply, e.g. in animal intestines. Arthrobacters are notorious for their longevity in a starved or desiccated state. Studies on Arthrobacter globiformis NCIB 10683, an acid forest soil isolate, suggested it belonged to the first group so this was investigated further.

Theoretical models were deduced describing maintenance and viability in continuous culture and tested using A. globiformis in glucose-limited cultures. Slight differences were found in parameter values depending on the model assumptions: one gave the specific maintenance rate to be $0.0105h^{-1}$ at $25^{o}C$, comparable to other soil microorganisms. The maximum yield factor, 0.602g (g glucose)$^{-1}$, indicated efficient substrate utilization. Deviations from a constant maintenance rate occurred at dilution rates below $0.025h^{-1}$, becoming $0.003h^{-1}$ at $0.01h^{-1}$. It also decreased with temperature becoming $0.002h^{-1}$ at $D = 0.02h^{-1}$ and $10^{o}C$. Viability measurements indicated a very low minimum growth rate of $\leqslant 0.001h^{-1}$.

Determinations of the saturation constant for glucose gave $\leqslant 2$ mg l^{-1} showing A. globiformis to have a high substrate affinity.

Cells grown at low rates could well have a composition similar to that in soil. The levels of protein, total carbohydrate, RNA, DNA, lipid and cell wall, mean cell mass and mean cell size were measured at various dilution rates.

The composition of carbon-limited cells changed little apart from a decrease in total carbohydrate (intracellular polysaccharide?) with dilution rate. Slowly-grown cells have "extra" RNA, in excess of that required for protein synthesis. The cell wall content was high (about 30% w/w). Cocci grown at a dilution rate of $0.01h^{-1}$ had twice the mean cell mass of those at $0.1h^{-1}$. Also an increase in mean cell specific gravity was observed suggesting that the former cells have a more compact structure.

A comparative study of nitrogen-limited cells showed accumulation of an intracellular polysaccharide (of glucose): up to 65%. As the dilution rate was decreased, this resulted in an increase in cell yield, mean cell mass, mean cell size and mean cell specific gravity. These enlarged cells are equivalent to the "cystites" produced in old batch cultures except that they were undergoing normal division. The viability measurements were higher than for carbon-limited populations.

Chemostat-grown bacteria were starved in shake flasks at 25°C. A carbon-limited population (dilution rate = $0.01h^{-1}$) was 73% viable after 56 days, showed a 34% loss of carbohydrate,

32% of protein, 43% of RNA but little decrease in DNA. The major substrate for endogenous metabolism was protein. RNA turnover was only significant after 7 days when the $Q[O_2]$ had fallen to about 1.*

A nitrogen-limited population (dilution rate = $0.01h^{-1}$) showed a 37% loss of carbohydrate, about 20% of protein, 24% of RNA, but no change in DNA after 20 days, when the viability was at least 44%. The intracellular polysaccharide was the major endogenous substrate.

Starvation of nitrogen-limited cells grown at $0.1h^{-1}$ resulted in a rapid accumulation of carbohydrate from the glucose in the medium over 2 days. After 38 days this had been completely degraded with no loss of protein, a slight loss of RNA and a 20% increase in DNA. The viability fell to 42% and the $Q[O_2]$ to 6* indicating a high rate of endogenous metabolism.

Measurements of cell weight losses during starvation of the various populations supported an increase in an unmeasured constituent, possibly cell wall material.

The yellow pigment of A. globiformis was shown to be a carotenoid and, by comparison with a white mutant, to confer photoprotection.

It was concluded that the endogenous metabolism of carbon-limited A. globiformis is low and contributes to its

* $Q[O_2]$ values in $\mu l\ mg^{-1}\ h^{-1}$

longevity in soil. The increase in mean cell mass at low growth rates may aid this. Nitrogen-limited populations survive less well in spite of the polysaccharide reserve, probably due to the higher rate of endogenous metabolism.

CHAPTER 1. INTRODUCTION

ENDOGENOUS METABOLISM IN BACTERIA AND ITS RELATIONSHIP TO SURVIVAL

Definition of Endogenous Metabolism

Endogenous metabolism has been defined as the sum of metabolic processes occurring within a living cell in the absence of an exogenous energy supply (Lamanna, 1963; Dawes and Ribbons, 1964) and many writers have used it in this restricted sense (Herbert, 1958). However, from the nature of these processes it is certain that they must continue when an exogenous energy supply is present and when the cell is actively growing and dividing. Thus it might be more appropriate to regard endogenous metabolism as the sum of metabolic processes within a cell not directly related to growth and division, regardless of whether the cell is dividing at its maximum rate or is starving, even to the extent of being technically 'dead', i.e. incapable of division.

Endogenous metabolism may be quantified by considering 'maintenance energy' (Mallette, 1963) which is the energy required for processes other than growth. Another practical measure is 'endogenous respiration' which refers to the oxygen consumed in endogenous metabolism. It is only applicable to aerobic conditions but few

studies at all have been made of endogenous metabolism under anaerobic conditions.

The need for Endogenous Metabolism

The reasons for a cell requiring endogenous metabolism have been deduced rather than proven, with a few exceptions. It is suggested that the energy generated in endogenous metabolism meets the need for osmo-regulation including the maintenance of intracellular pH, the turnover of cell wall material, proteins and nucleic acids, motility, sporulation, luminescence and the need to mobilize endogenous reserves of carbon, or other elements, required in the resynthesis of degraded cell constituents. In some organisms these reserves might be used as a source of reducing power (Dawes and Ribbons, 1962).

Some work has indicated that at least part of the energy released in endogenous metabolism is wasted. This is particularly so where an energy supply is in excess or where the organism is under some form of environmental stress. Energy released from the oxidation of reserve materials or an exogenous source ultimately occurs in the form of high energy phosphate bonds in ATP. The energy so stored is then used to meet the energy requirements of the organism and the degree to

which these two processes are linked is referred to as the degree of 'coupling'. Where energy is wasted, this is referred to as 'uncoupling' (Senez, 1962). This is presumably due to the spontaneous hydrolysis of phosphate bonds in ATP 'uncoupled' to any other metabolic reaction, the energy being lost as heat. It has been suggested that this uncoupling can be useful to an organism such that energy in the form of ATP is readily available to be channelled into some process when the need arises, thus being analogous to a 'slipping clutch' (Tempest, 1975).

Measurement of Endogenous Metabolism

Measurement of maintenance energy or endogenous respiration can be made in either growing or non-growing populations. Endogenous respiration is measured by manometry, i.e. by estimation of oxygen uptake. In the absence of exogenous substrates it is presumed that oxidation of endogenous substrates takes place. The carbon dioxide evolved during respiration may also be measured, preferably by using cells uniformly labelled with carbon-14.

A drawback of measuring the rate of production of carbon dioxide is that it cannot be related to the energy generated by the endogenous metabolism unless

the substrates used are known. Measurements of R.Q. values can give a useful indication as to what these substrates are. An alternative method is to monitor directly the decrease in levels of endogenous substrates. This could be measured by the decrease in cell weight if only one known substrate was being utilized. This is applicable in some cases but usually more than one cell constituent is degraded even if it is sequential and therefore they have to be measured individually. In assessing the energy produced, any losses to the medium, due to leakage or excretion, of metabolic by-products or products of degradation, particularly of nucleic acids, need to be taken into account.

The energy released during endogenous metabolism finally ends up as heat and so micro-calorimetry is a convenient way of measuring endogenous metabolism (Forrest, 1972).

Cells do not always grow in the presence of an exogenous energy or carbon source due to the requirement for another factor but the measurement of endogenous metabolism is feasible providing the utilization of both exogenous and endogenous substrates are taken into account. In some experiments an external carbon source has been added but at a concentration too low to permit growth (McGrew and Mallette, 1962).

The endogenous respiration of growing cells can also be measured by comparing their behaviour at more than one growth rate. Several workers have measured oxygen uptake and carbon dioxide release over a range of growth rates in a chemostat and extrapolated the values obtained to zero growth rate. This is assumed to represent the endogenous respiration which is also assumed to be constant and independent of the growth rate. In most cases the data obtained supports this assumption although the measurements are usually made over moderate to high growth rates and are not sensitive enough to detect small changes in endogenous metabolism. Another method first used by Marr, Nilson and Clarke (1963) is to measure the yield of cells over a range of growth rates in a chemostat. As the growth rate is decreased the proportion of substrate diverted to endogenous metabolism becomes greater resulting in less being available for the production of cell mass and so the cell yield decreases. From these measurements the maintenance energy can be calculated and details of the theory will be found later.

Relationship to Survival

Endogenous metabolism is important for the survival of cells during starvation where starvation is defined as the absence of one or more factors necessary for growth

and division although it is usually taken to mean the absence of an energy source. However, if endogenous metabolism during starvation of some factor other than an energy source is considered, then the picture is complicated since both exogenous and endogenous substrates are available.

Endogenous metabolism can also be important under various stress conditions other than or as well as starvation if combating the stress requires the input of energy (e.g. Watson, 1970).

Even in conditions that are 'stress-free' apart from starvation it is evident that a cell will require energy to meet the needs of protein turnover, etc., and this is provided by endogenous metabolism (Postgate, 1967). Theoretically the organism will remain viable as long as the energy input can be met. If, during starvation, a cell can minimize its endogenous metabolism, then with a limited energy supply it will maximize its survival.

The assumption that endogenous metabolism is constant is not necessarily true. Tempest and Herbert (1965) have shown that endogenous metabolism varies with growth rate in continuous culture using the yeast, Torula utilis (NCYC 321). Also during starvation oxygen uptake rates ($Q[O_2]$ values) may decrease rapidly before stabilizing at a low value with no change in viability (Boylen and Ensign, 1970a). The

reasons for such a change are not clear but may indicate a shutting down of cell processes. Thus, a change in membrane permeability could reduce energy requirements for osmo-regulation. Slow-growing and resting cells may not need stringent pH control. Loss of inducible enzymes has been observed such as the loss of β-galactosidase activity from starved Escherichia coli (Ryan, 1959). Motility or luminescence could be dispensed with without endangering cell integrity. The degree of coupling between catabolic and anabolic processes could become more complete. Bacteria which can form spores obviously have a mechanism for reducing their endogenous metabolism due to the special nature of the spore coat and protoplast. Respiratory activity in spores is negligible and the turnover rate of endogenous reserves is so small that they would last for hundreds of years (Sneath, 1962; Murrell, 1967).

Problem of Viability. Viability is usually defined as the ability of a cell to divide under favourable conditions. The relationship between survival and viability measurements has been reviewed by Postgate (1967). He recognized the distinction between 'viable cells' - able to divide, 'senescent cells' - functional biological entities incapable of division, and 'moribund cells' - those in which the osmo-regulation mechanism had broken down. 'Senescent cells' are thus able to metabolize endogenous substrates. Their detection is experimentally

difficult but studies of scarring of yeast cells, where a certain maximum number of scars on a cell stops further reproduction, suggest that these cells are senescent (Beran, Malek, Streiblova and Lieblova, 1967). This has less relevance to most bacteria which undergo binary fission and do not get 'older' in the way a budding yeast does. Microorganisms whose division has been arrested by inhibitors could be considered to be in a state of forced senescence.

The assessment of viability is difficult. Postgate (1967) discussed several indirect methods such as the use of vital stains but none were considered to be satisfactory. The best method for populations with a viability of 5-100% is the slide culture technique (Postgate, Crumpton and Hunter, 1961). Here the ratio of 'viable' to 'non-viable' organisms is counted directly by microscopic observation after growth on a suitable recovery medium thus eliminating the errors extant in traditional total and plate counts (Postgate and Hunter, 1962). The term 'non-viable' will include both 'senescent' and 'moribund'. An additional advantage of this method is that the degree of recovery of a particular cell may be observed which may range from swelling-up or germination to the formation of a large colony. Also the variation in the degree of recovery within the population can be seen.

Substrates involved in Endogenous Metabolism

The intracellular substrates consumed during endogenous metabolism will now be considered.

Amino Acids. In Micrococcus luteus (formerly Sarcina lutea) half the amino acid pool is used during aeration of a starved culture but no relationship between the pool size and the capacity for survival has been detected (Ribbons and Dawes, 1963).

Protein. Protein utilization in starved bacteria occurs to a limited degree after a primary substrate has been depleted. There is usually a concomitant release of ammonium ions. Strange, Dark and Ness (1961) showed that in tryptone-glucose grown Klebsiella pneumoniae* containing 21% carbohydrate, the glycogen is depleted in 25 h with a small change in protein content but then further protein and some RNA degradation occurs with the release of ammonium ions. In cells containing 4-6% carbohydrate, grown in tryptic meat broth or in a defined medium, protein and RNA are degraded with little change in carbohydrate content. Pseudomonas aeruginosa, which has no reserve materials, when previously fed with labelled proline, liberated labelled carbon dioxide during starvation showing protein degradation (Campbell, Gronlund and Duncan, 1963). Protein-rich K. pneumoniae cells maintained their viability whilst using their protein (Strange et al., 1961).

* formerly Aerobacter aerogenes

RNA. RNA can play an even greater role than protein as a substrate for endogenous metabolism. Strange et al. (1961) showed that stationary-phase K. pneumoniae from a defined medium lost 40% RNA in 70h but maintained 70% viability. The main products were ammonia, inorganic phosphate and free bases. Up to 50% of the RNA of bacteria may be lost without any harmful effect.

There are no records of DNA, structural lipid or cell wall material being used during starvation.

Reserve Materials. Many bacteria possess one or more forms of reserve material. These take the form of carbohydrate, polyglucoses resembling glycogen and generally called by that name, or lipid, notably poly-β-hydroxybutyrate (PHB). Some organisms can accumulate sulphur which is later used as an energy source. Polyphosphates, at one time thought to be concerned with energy storage, may be more related to a reserve for phosphorus (Dawes and Senior, 1973). Studies on polyphosphates have shown no benefit in survival.

Glycogen is a substrate for endogenous metabolism and therefore helps to maintain viability in a number of organisms. Strange et al. (1961) showed glycogen utilization in K. Pneumoniae and that cells with a higher level maintained almost 100% viability for a longer period. The maintenance of viability did not rely solely on the presence of glycogen, for although it had

disappeared within 25h from tryptone-glucose grown cells, they remained over 90% viable after 70h. A similar situation occurs in Escherichia coli where glycogen serves as a primary endogenous substrate. Depletion is even more rapid, occurring within 3h, whilst viability is constant for 12h. The glycogen depletion precedes degradation of protein and the release of ammonia. Experiments suggest however, that the glycogen prevents net protein degradation but not protein turnover as labelled protein gives rise to labelled carbon dioxide during this initial period. Thus glycogen may serve as a reserve of carbon skeletons and not of energy (Dawes and Ribbons, 1965). Due to its very rapid utilization, glycogen cannot play much part in increasing survival in E. coli. In contrast, van Houte and Jansen (1970) have shown that during the utilization of glycogen by Streptococcus mitis over 20h, viability is maintained but thereafter it decreases rapidly as it does in glycogen-deficient cells from the beginning of starvation. Starved suspensions of glycogen-rich, logarithmic-phase cells of an Arthrobacter sp. maintained a high viability over 20 days whilst the glycogen was degraded. After 5 days the endogenous respiration had fallen to a very low level (Zevenhuizen, 1966). The presence of glycogen (or more correctly an oligosaccharide) appears to have a deleterious effect in Micrococcus luteus.

Glucose-peptone grown cells had a viability of 10-20% after 30h starvation whereas peptone grown cells remained over 90% viable (Burleigh and Dawes, 1967).

PHB is also used as a substrate for endogenous metabolism and increases longevity in starved populations. Macrae and Wilkinson (1958) showed this for Bacillus megaterium and found that B. cereus cells rich in PHB had $Q[O_2]$ values three times higher than cells poor in PHB. Cells of Paracoccus halodenitrificans (formerly Micrococcus halodenitrificans) containing 50% PHB maintained 100% viability over 100h whilst the PHB was degraded and the endogenous respiration remained high : $Q[O_2] = 40 \mu l\ O_2\ mg^{-1}\ h^{-1}$. Upon PHB depletion the viability and $Q[O_2]$ fell rapidly. Cells having only 10% PHB showed an immediate decrease in both viability and $Q[O_2]$ reaching low levels after 50h (Sierra and Gibbons, 1962). Hippe (1967), using Alcaligenes eutrophus (formerly Hydrogenomonas H16), showed that a high PHB content prevented net degradation of nitrogen-containing compounds and the release of ammonia during metabolism of 50% of the PHB over 84h. During this period the $Q[O_2]$ fell from 18 to 2.7. In PHB-poor cells, after rapid PHB degradation, the cells maintained 70% viability after 70h whilst the protein level decreased. Again in Azomonas agilis (formerly Azotobacter agilis), PHB-rich cells maintained high viability over 48-72h followed by a decrease; PHB-poor cells lost viability from 0h down

to 10-30% over 72h. However succinate-grown cells, with a lower PHB content than glucose-grown cells, degraded PHB at a slower rate and maintained viability longer (Sobek, Charba and Foust, 1966).

It can be concluded that the retention of viability can depend on the initial level of reserve material but that the rate of utilization, reflected in the $Q\ [O_2]$ values, is important as a low endogenous metabolic rate prolongs viability even when the quantity of reserve material is small.

Role of magnesium. A number of studies, summarized by Strange and Hunter (1967), have indicated that magnesium ions can play an important role in enhancing bacterial survival. It is suggested that the presence of magnesium has a stabilizing action on ribosomes and thus on RNA degradation, that it influences metabolism so as to reduce the effects of substrate-accelerated death (Postgate and Hunter, 1964), and that it has a stabilizing effect on the permeability control mechanisms which are otherwise susceptible to cold shock.

Table 1 summarizes the results of some studies on endogenous metabolism and survival in starved cultures. The organisms are listed in approximate order of increasing survival ability which depends not only on the species but also on the growth conditions.

Table 1. Viability and respiration rate changes in populations of starved bacterial suspensions.

Organism	Conditions	Time period h	Viability change over time period, %	Q (O_2) values $\mu l\ mg^{-1}\ h^{-1}$
Escherichia coli	(glycogen used in 3h)	12	constant (then rapid decrease)	
Streptococcus mitis	glycogen-rich	20	constant (then rapid decrease)	
Micrococcus luteus	with oligosaccharide	30	ca. 100 to ca. 15	
Paracoccus halodenitrificans	10% PHB	50	100 to ca. 0	40 to 5
Azomonas agilis	PHB -poor	72	100 to ca. 20	
Klebsiella pneumoniae	from tryptic meat, 4-6% carbohydrate	70	100 to 60	
"	minimal medium, 4-6% carbohydrate	70	ca. 100 to 70	
M. luteus	without oligosaccharide	30	ca. 100 to 90	
Alcaligenes eutrophus	PHB-poor	95	100 to 70	17 to 3 (20h)
K. pneumoniae	tryptone-glucose, 21% carbohydrate	70	ca. 100 to 90	
A. agilis	PHB-rich	48-72	ca. 100 (then rapid decrease)	
P. halodenitrificans	50% PHB	100	100 (then decrease)	40 (then decrease)
A. eutrophus	PHB-rich	84	constant	18 to 2.7
Arthrobacter sp.	Glycogen-rich	480	constant	low level after 120h

Measurements of maintenance energy

The examples given so far are concerned with endogenous metabolism and survival in populations held without an exogenous energy supply. Numerous workers have measured maintenance energy in growing populations using continuous culture. The results are presented in table 2. Maintenance energy is expressed in two ways : firstly, as a maintenance coefficient (Pirt, 1965) which is the mass of substrate required per mass of cells per unit time to supply the energy for maintenance, and secondly, as a specific maintenance rate, expressed in units of reciprocal time, being the product of the maintenance coefficient and the true growth yield. The specific maintenance rate can be considered to be the amount the growth rate is reduced by due to the diversion of energy from growth to maintenance (Powell, 1967).

Relationship to environment

It has been concluded that a low level of endogenous respiration is related to longevity and that the possession of intracellular substrates for endogenous metabolism aids survival. A low maintenance requirement might also be expected to aid survival. It is instructive therefore to consider the natural environments of these organisms and what relation their endogenous metabolism has to them. Table 3 gives a list of the organisms used in tables 1 and 2 together with their habitats.

Table 2. Maintenance parameters and growth yields of various microorganisms grown in continuous culture.

Organism	Temperature °C	Limiting substrate	Maintenance coefficient g substrate g dry wt^{-1} h^{-1}	Specific maintenance rate, h^{-1}	True growth yield g dry wt g substrate^{-1}	Comment
Pseudomonas fluorescens[1]	30	glucose	0.445	0.233	0.512	Assuming 1 mole glucose gives 3 moles ATP
Lactobacillus casei[2]	37	glucose	0.225	0.091	0.405	anaerobic
Lipolytic bacterium[3]	39	glycerol	0.300	0.090	0.30	
Acinetobacter calcoaceticus[4]	35	ethanol	0.11	0.0853	0.775	
Klebsiella pneumoniae[3]	37?	glycerol	0.076	0.042	0.55	
Enterobacter cloacae[3]	37	glucose	0.094	0.041	0.44	
"	"	"	0.473	0.039	0.083	anaerobic
Azotobacter vinelandii[5]	30	oxygen	0.144	0.037	0.256	nitrogen-fixing, glucose as energy source
Escherichia coli[6]	30	glucose	0.055	0.028	0.51	
E. coli strain P3[7]	30	glucose	-	0.028	-	feeding experiment
Saccharomyces cerevisiae[8]	20	glucose	0.360	0.027	0.076	anaerobic, medium contains 1.0 M sodium chloride
E. coli strain P3[7]	30	glucose	0.064	0.025	0.39	
E. coli strain ML30[7]	30	glucose	-	0.018	-	feeding experiment
Pseudomonas sp[9]	30	glutamate	0.053	0.015	0.28	anaerobic respiration, nitrate electron acceptor
Aspergillus nidulans[10]	30	glucose	0.029	0.014	0.48	
Pseudomonas sp[9]	30	glutamate	0.029	0.011	0.39	
Penicillium chrysogenum[11]	25	glucose	0.0216	0.0097	0.45	
S. cerevisiae[12]	30	glucose	0.014	0.0071	0.505	
E. coli strain P3[7]	15	glucose	-	0.005	-	feeding experiment
S. cerevisiae[8]	20	glucose	0.036	0.0037	0.103	anaerobic

Table 2. (continued) References.

1 Mennett and Nakayama (1971)

2 DeVries *et al.* (1970)

3 Pirt (1965)

4 Abbot, Laskin and McCoy (1974)

5 Nagai and Aiba (1972)

6 Schulze and Lipe (1964)

7 Marr *et al.* (1963)

8 Watson (1970)

9 Koike and Hattori (1975)

10 Bainbridge *et al.* (1971)

11 Righelato *et al.* (1968)

12 Nagai and Aiba (1972), data of von Meyenburg (1969)

Table 3. Habitat or site of isolation of microorganisms given in tables 1 and 2.

Acinetobacter calcoaceticus	soil and water, also isolated from man.
Alcaligenes eutrophus	soil and water.
Arthrobacter sp.	isolated from soil.
Aspergillus nidulans	soil.
Azomonas agilis	limited to fresh water.
Azotobacter vinelandii	soil and water.
Enterobacter cloacae	found in feces, sewage, soil and water, pathological material.
Escherichia coli	lower part of intestine of warm-blooded animals.
Klebsiella pneumoniae	widely distributed in nature, soil, water, grain, and normally found in intestinal canal.
Lactobacillus casei	dairy products and dairy environments, human intestine.
Lipolytic bacterium	rumen of sheep.
Micrococcus luteus	common in soil, dust, water, skin of man and other animals, non-pathogenic.
Paracoccus halodenitrificans	meat-curing brines, presumably widely distributed in natural and artificial brines.
Penicillium chrysogenum	soil.
Pseudomonas fluorescens	soil and water.
Pseudomonas sp.	common in soil, water and marine environments.
Saccharomyces cerevisiae	surface of grapes, overwinters in soil.
Streptococcus mitis	human saliva, sputum and feces.

These have been largely taken from Buchanan and Gibbons (1974).

Microorganisms found associated with human or animal bodies would be expected to have a food supply of fairly short periodicity (Koch, 1971) and so would only be required to survive for short periods. Thus Escherichia coli and Streptococcus mitis have high rates of turnover of endogenous substrates and rapid loss of viability in a matter of hours. Some micro-organisms are associated with animal bodies but are also found in other environments although the importance of the latter is difficult to ascertain : Micrococcus luteus and Klebsiella pneumoniae have a moderate survival in starved suspension. Microorganisms from habitats with less frequent energy inputs or with constant but very low inputs would need to survive longer and to have a low endogenous metabolism. This is seen in Paracoccus halodenitrificans, Alcaligenes eutrophus and Azomonas agilis which have survival times, depending on the initial state of the cells, ranging 1-4 days or more. Arthrobacter spp. show even greater longevity and this will be dealt with in more detail later.

A comparison of maintenance requirements is not so straightforward. Here the specific maintenance rates must be compared since the values for the maintenance coefficients are dependant on the particular substrate used.

Temperature must also be taken into account since the specific maintenance rate depends on this. The micro-organisms associated with animals, Lactobacillus casei, the Lipolytic bacterium, Klebsiella pneumoniae, Enterobacter cloacae (formerly Aerobacter cloacae) and Escherichia coli have high values : above 0.02 h^{-1}. Azotobacter vinlandii also has a high value but this is probably due to its need for a high rate of endogenous respiration to prevent oxygen inhibition of its nitrogenase system (Nagai and Aiba, 1972). The value in Acinetobacter calcoaceticus may be due to the high incubation temperature or to the nature of the substrate, ethanol. The high value for Saccharomyces cerevisiae of 0.027h^{-1} is due to the presence of 1.0 M sodium chloride in the medium. The very high value for Pseudomonas fluorescens is surprising although Palumbo and Witter (1969) using a psychrophilic strain also found the specific maintenance rate to be high : 0.167 h^{-1}. However both sets of workers used absorbance measurements rather than direct cell yield determinations which may have been influenced by changes in cell size with growth rate and so overestimated the values for maintenance. Alternatively this might be a reflection of the zymogenous character of this species.

The remaining organisms associated with soil or water, the Pseudomonas spp.(formerly Ps. denitrificans, nomina incertae sedis), Aspergillus nidulans and Penicillium

chrysogenum have values below 0.02 h^{-1}. S. cerevisiae also has a very low specific maintenance rate; this may be due to the fact that this organism probably overwinters in the soil environment.

In view of what has been discussed it is apparent that any ecological study of an organism living in a nutrient-poor environment such as soil should take into account the endogenous metabolism of that organism.

STUDIES ON ARTHROBACTER GLOBIFORMIS NCIB 10683

Taxonomic position of this strain

The organism used in this study was originally isolated from the A_1 horizon of an acid pine forest soil by Lowe (1969). The soil has been described by Goodfellow, Hill and Gray (1968).

The organism, originally denoted as A69, was found to lie on a minimum spanning tree within a group of Arthrobacters adjacent to the type strain Arthrobacter globiformis NCIB 8907 with which it has a matching coefficient (Sokal and Michener, 1958) of 92.6% and a similarity coefficient (Sneath, 1957) of 83.8% (Lowe, 1969; Lowe and Gray, 1972). It has since been designated as Arthrobacter globiformis NCIB 10683 and a list of its properties from the data of Lowe (1969) are given in appendix I. It is therefore typical of a group of

organisms abundant in soils of various kinds and which displays the ability to withstand a range of environmental conditions. For a summary of the properties of the genus see Keddie (1974) and Duxbury (1973).

The taxonomic state of the Arthrobacters, often described as such solely on the fact that the organism undergoes rod to coccus morphogenesis, is uncertain. Originally placed in the Corynebacteriaceae, recent numerical taxonomy studies have indicated that the group Arthrobacter is distinct from other coryneforms although the taxon remains loose (Bousfield, 1972; Yamada and Komogata, 1972; Jones, 1975).

Success of Arthrobacter in soil.

Both Keddie (1974) and Hagedorn and Holt (1975) have indicated that Arthrobacter isolates can use a wide range of organic compounds although this ability can be lost from strains kept in culture as judged from the limited range of type strains. It is suggested that this flexibility is one of the contributory factors to the success of the Arthrobacters in soil. Lowe and Gray (1972) did not find such a distinction between soil isolates and the type strains. It is to be concluded that this distinction will depend on the type strain and also on the nature of the tests applied.

Survival. Another factor implicated is the success of

Arthrobacters is the ability to survive longer than other vegetative soil microorganisms. Mulder and Antheunisse (1963) showed that Arthrobacters could survive in desiccated soil for up to ten months whereas other vegetative cells disappeared after two months. Spore-forming organisms, the Actinomycetes and Bacillus spp. were able to survive much longer. Robinson, Salonius and Chase (1965) studied the survival of Arthrobacter spp. and Pseudomonas spp. inoculated into γ -sterilized soil and dried. There was no change in numbers of Arthrobacters, after the initial drying, for a period of one month, whereas a large drop occurred in the numbers of Pseudomonas. Recent soil isolates were found to be more resistant than the type strains A. globiformis and Ps. fluorescens. Chen and Alexander (1973) determined the numbers and kinds of bacteria surviving under dry conditions in two soils and found that Arthrobacters were the only vegetative cells to survive up to 90 days. Boylen (1973) using Arthrobacter crystallopoietes (now called A. globiformis ATCC 15481) found that, after the initial drying of cells inoculated into sand cultures, no change in viability occurred for up to 6 months.

Chen and Alexander (1972) studied the survival of soil microorganisms in a carbon-free solution and found that, next to the spore-formers, Arthrobacters were among the most persistant. Boylen and Ensign (1970a)

found that A. globiformis ATCC 15481 when starved in buffer remained 100% viable for 30 days and was 65% viable after 60 days. It is concluded that Arthrobacter spp. have an almost unique survival ability in both dry and wet conditions and in pure and mixed cultures.

Lowe and Gray (1973) looked into the growth of soil isolates reinoculated into sterile, pine-forest soil. The isolates, including an Arthrobacter sp. denoted as A21 and which is phenetically very similar to A. globiformis NCIB 10683, were found to grow and remain at high levels after 7 days in the alkaline C horizon (pH 8.1) and the A_1 horizon when amended with calcium carbonate or potassium hydroxide (pH 7.5). However, in the unamended A_1 horizon (pH 4.3) the cells rapidly disappeared. In answer to the problem of why bacteria isolated from the acidic A_1 horizon were not able to grow, or even survive, when reintroduced, it was suggested that cells grown on laboratory media were physiologically different from cells growing slowly in the nutrient-deficient soil environment.

Physiology of chemostat-grown A. globiformis. Luscombe (1972) followed up these studies by looking into the physiology of A. globiformis NCIB 10683 using continuous culture as a means of producing cells whose state might more closely represent that found in soil than cells grown on laboratory batch media. It was found that this

organism behaves according to the simple theory of continuous culture and that the characteristic rod-coccus morphogenesis was primarily growth rate dependant, rods occurring only when the growth rate approached near to the maximum possible at that temperature (Luscombe and Gray, 1971). It was also shown that survival under stress conditions : low water availability (a_w =0.90), low pH (4.1) and elevated temperature (48°C) could be affected by the conditions of cell-growth, the most important factor being whether the cells were grown in nitrogen or carbon-limited media. However, loss of viability under the various stresses was rapid : 99% loss occurring in ¼-12 hours. Starvation of carbon-limited cells in phosphate buffer at pH 7.0 showed survival comparable to A. globiformis ATCC 15481; the viability was still 15% after 56 days for rod cells and after 70 days for coccoid cells.

Cell wall of A. globiformis. Since the cell wall of an organism represents the barrier between it and the environment it was considered that its nature could play a significant role in the survival of Arthrobacter. Duxbury (1973) therefore madea comparative study of the cell walls of rods, cocci and so-called cystites (produced in media of high carbon: nitrogen ratio) of A. globiformis NCIB 10683. Rates of autolysis of all three forms were very low particularly when compared to pathogenic microorganisms. The cell wall was found to consist of

ca. 60% of a polysaccharide of glucose, galactose and rhamnose and 40% mucopeptide. It was suggested that the short length of the interpeptide bridges conferred resistance to autolysis and that the polysaccharide might also add mechanical strength or protect the mucopeptide from external enzymatic attack. The wall was two-layered and ca. 30nm thick. Direct observation of cell growth showed that division was by budding, giving the concept of 'old' and 'young' cells which was confirmed by electron micrographs.

AIMS OF PRESENT WORK

It has been shown that endogenous metabolism can play a large part in the survival of microorganisms and also that Arthrobacter spp. have the ability to survive for exceptional periods of time under starvation conditions. It is reasonable to propose that Arthrobacter spp. have a very conservative endogenous metabolism. This has already been indicated by Zevenhuizen (1966), Boylen and Ensign (1970a, 1970b) and Luscombe (1972).

The aims of this study were to investigate further the endogenous metabolism of Arthrobacter globiformis NCIB 10683. This was to be done by : firstly, investigating the maintenance energy using continuous culture; secondly observing how the organism altered in composition at different growth rates produced in the chemostat where the slower growth rates might approximate to conditions

found in the natural soil environment; and thirdly, by observing the change in composition of cells under non-growing conditions to determine what substrates are used for endogenous metabolism.

It is generally assumed that bacteria in the soil environment are carbon-limited or perhaps, more correctly, energy-limited. This is not necessarily true; other nutrients could be limiting all, or at least some part of, the time. Nitrogen is one of the most likely limitations, particularly as the natural soil, the A_1 horizon, has a carbon-nitrogen ratio of 32:1. It was considered instructive therefore to perform the studies using both carbon and nitrogen limited conditions.

CHAPTER 2. MAINTENANCE ENERGY

THEORY

Since the original formulations of the theory of continuous cultivation by Monod (1950) and Novick and Szilard (1950) several workers have modified and extended it to take into account certain experimental observations and systems other than the single-stage chemostat. Two important modifications, which will be discussed here, are concerned with endogenous metabolism and the effect of part of the chemostat population being non-viable. The equations, with some extensions, are based on those given by Herbert (1958), Marr et al. (1963), Pirt (1965), Powell (1967) and van Uden (1969). These workers used different symbols for the same concepts but in the following discussion the symbols recommended in the 2nd International Symposium on the Continuous Cultivation of Microorganisms (Malek, Beran and Hospodka, 1964) have been adhered to as far as possible. These are listed on a removable sheet in the wallet on the back cover and are also given in appendix 2.

Models of maintenance energy in continuous culture

In continuous culture the principal parameters controlled by the experimenter are the medium composition, the temperature and the dilution rate. All other parameters are determined by the biological nature of the organism grown. The dilution rate is given as :

$$D = F/v$$

and will determine the rate of inflow and outflow of any substance in the liquid phase. Considering the change in total cell biomass :

$$dx^T/dt = \mu x^v - Dx^T$$

since only the viable population contributes to growth. If a steady state has been reached :

$$dx^T/dt = 0$$

and

$$\mu x^v = Dx^T \qquad (1)$$

Considering the change in "non-viable" biomass :

$$dx^{nv}/dt = \mu_d x^v - Dx^{nv}$$

In the steady state :

$$\mu_d x^v = Dx^{nv} \qquad (2)$$

From (1)

$$\mu x^v = D(x^{nv} + x^v)$$

so

$$\mu x^v = \mu_d x^v + Dx^v$$

$$\mu = \mu_d + D \qquad (3)$$

Considering the change in limiting substrate concentration:

$$ds/dt = D\,S_r - (q^v x^v + q^{nv} x^{nv} + Ds)$$

In the steady state:

$$D/(S_r - s) = q^v x^v + q^{nv} x^{nv} \qquad (4)$$

Now $q^v x^v$ is the rate of consumption of substrate by the viable population per unit volume and this can be

split into the quantity used directly for growth and that used in endogenous metabolism. Hence :

$$q^v x^v = \mu x^v / Y_{max} + q_e^v x^v$$

q_e^v is the equivalent of Pirt's maintenance coefficient, m, and may be expressed as μ_e / Y_{max} where μ_e is the specific maintenance rate (identical to Marr's 'a' and Herbert's 'k'). Therefore :

$$q^v = \mu / Y_{max} + \mu_e / Y_{max}$$

also:

$$q^v_{max} = \mu_{max} / Y_{max} + \mu_e / Y_{max}$$

Substituting into (4) :

$$D(S_r - s) = \mu x^v / Y_{max} + \mu_e x^v / Y_{max} + q^{nv} x^{nv}$$

Using (1) and (2) it is possible to substitute x^T for x^v and x^{nv} to give :

$$x^T = \frac{Y_{max}(S_r - s)\mu D}{D(\mu + \mu_e) + Y_{max} q^{nv} \mu_d}$$

Since

$$x^T = Y S_r$$

$$Y = \frac{Y_{max}(1 - s/S_r)\mu D}{D(\mu + \mu_e) + Y_{max} q^{nv} \mu_d} \qquad (5)$$

<u>Substrate concentration</u>. The significance of s will now be discussed. According to Monod (1950) the rate of consumption of substrate by a microorganism can be related to the external substrate concentration by :

$$q^v = q^v_{max} \, s/(K_s + s) \qquad (6)$$

[Note that,

$$\mu = \mu_{max}\, s/(K_s + s)$$

does not take into account the substrate diverted to endogenous metabolism.] Powell (1967) has considered several other formulations which might give a closer fit to experimental findings. However, at the usual values of S_r the effect on the predicted steady state value of Y will be very small although the predicted steady state value of s would depend on the formulation adopted. Substituting (6) into (5) leads to:

$$Y = Y_{max}\left[1 - \frac{K_s(\mu + \mu_e)}{S_r(\mu_{max} - \mu)}\right] \frac{\mu D}{D(\mu + \mu_e) + Y_{max} q^{nv} \mu_d} \qquad (7)$$

This equation is clumsy but a useful close approximation can be made by ignoring the decrease in yield due to incomplete consumption of the substrate since in most experimental systems S_r is much greater than K_s. Large deviations will only occur as μ tends to μ_{max}. Hence :

$$Y = Y_{max} \frac{\mu D}{D(\mu + \mu_e) + Y_{max} q^{nv} \mu_d} \qquad (8)$$

<u>Importance of "non-viable" biomass</u>. So far q^{nv}, the consumption of substrate by the "non-viable" biomass, has not been dealt with and here experimental evidence which would give indication as to what this might be is lacking. The simplest assumption is that $q^{nv} = 0$ giving :

$$Y = Y_{max}\, \mu/(\mu + \mu_e)$$

or, from (3) :

$$Y = Y_{max}(D + \mu_d)/(D + \mu_d + \mu_e) \qquad (9)$$

Alternatively, q^{nv} might furnish energy for maintenance if the "non-viable" population is senescent rather than moribund, i.e.:

$$q^{nv} = \mu_e^{nv} / Y_{max}$$

giving :

$$Y = Y_{max} \frac{(D + \mu_d)\ D}{D(D + \mu_d + \mu_e) + \mu_d\ \mu_e^{nv}} \qquad (10)$$

and if $\mu_e^{nv} = \mu_e$:

$$Y = Y_{max}\ D/(D + \mu_e) \qquad (11)$$

This equation does not contain μ_d which means that the yield is independant of the specific death rate and hence of the viability of the population. It is therefore the same as those given by Marr et al. (1963) and Pirt (1965) who do not consider viability at all.

Van Uden (1969) used a factor "f" such that $q^{nv} = f\ q^{v}$, i.e. the rate of consumption by the "non-viable" biomass is a certain fraction of the rate of consumption by the viable biomass. Van Uden has reported establishing by a manometric technique that in a strain of Saccharomyces cerevisiae grown at superoptimal temperatures, f is greater than zero. It is worth noting that in this case q^{nv} will vary with the substrate concentration and so with the dilution rate. Also, unless the metabolic activity or rate of substrate uptake in "non-viable"

cells is greater than that of viable cells, which seems unlikely, f will not be greater than one. Van Uden does not consider the fate of this consumed substrate but one could envisage part going to maintenance. However, since maintenance is independent of the dilution rate, the remaining part will vary in magnitude with the dilution rate and it could be that the energy derived is wasted, i.e. the metablism is uncoupled. Putting f = 1 obtains :

$$Y = Y_{max}\, D/(D + \mu_d + \mu_e) \qquad (12)$$

An alternative to uncoupling is that the substrate consumed is converted into cell biomass. By definition a "non-viable" cell cannot divide, but it could increase in length or accumulate reserve materials. Obviously this cannot go on indefinitely but if the time period over which this occurs is comparable to the mean retention time within the chemostat, then a significant proportion of the "non-viable"biomass could be "growing" (see p. 32). If "non-viable" cells do grow then some modification to the biomass balance, equation (1), will be required. This may be indicated by putting μ^{nv} as the "growth" of "non-viable" cells. This gives :

$$Y = Y_{max}\, \frac{D(\mu - \mu^{nv})}{D(\mu + \mu_e) + \mu_e^{nv}\,\mu_d - \mu^{nv}(D + \mu_e)} \qquad (13)$$

(It can be easily seen that if $\mu^{nv} = 0$, (13) reduces to (10)). An interesting simplification of this is obtained if again $\mu_e^{nv} = \mu_e$:

$$Y = Y_{max}\, D/(D + \mu_e)$$

which is identical to equation (11). This means that μ^{nv} has no effect on the yield and, as before, nor does μ_d.

The models given are based on the assumption that the "non-viable" biomass is a homogeneous population, whereas in practice it is likely to be more heterogeneous than the viable biomass, since it will consist of moribund (completely dead) cells and cells in varying degrees of senescence. Also no consideration has been given to the release of nutrients from "non-viable" cells causing cryptic growth (Ryan, 1959; Postgate and Hunter, 1962) or alternatively the release of toxic substances which might inhibit the viable population.

Models used for testing. Of the above equations, three were chosen for testing with the experimental data, partly for their simplicity and partly because it was considered that the available data did not warrant any more detailed analysis. The equations, with the assumptions underlying them summarised, are as follows :

(9) $$Y = Y_{max}\, \frac{D + \mu_d}{D + \mu_d + \mu_e}$$

no consumption of substrate by "non-viable" biomass.

(11) $$Y = Y_{max}\, \frac{D}{D + \mu_e}$$

consumption of substrate by "non-viable" biomass for use in maintenance at same rate as viable biomass, or this, plus the fact that "non-viable" cells "grow."

(12) $Y = Y_{max} \frac{D}{D + \mu_d + \mu_e}$

consumption of substrate by non-viable biomass at same overall rate as viable biomass, part of which is used in maintenance and the remainder of which is wasted.

Equations (9) and (12) are the same as those given by van Uden (1969) with f=0 and f=1 respectively. However the implications behind all three have been extended.

All three equations are amenable to a form of double reciprocal plot in which the slope is equal to q_e^V (or μ_e/Y_{max}), the maintenance coefficient, and the intercept is equal to $1/Y_{max}$, i.e. :

(9) $\frac{1}{Y} = \frac{1}{Y_{max}} + \frac{1}{D + \mu_d} \cdot \frac{\mu_e}{Y_{max}}$

(11) $\frac{1}{Y} = \frac{1}{Y_{max}} + \frac{1}{D} \cdot \frac{\mu_e}{Y_{max}}$

(12) $\frac{D}{D + \mu_d} \cdot \frac{1}{Y} = \frac{1}{Y_{max}} + \frac{1}{D + \mu_d} \cdot \frac{\mu_e}{Y_{max}}$

Before equations (9) or (12) can be plotted it is necessary to know the values of μ_d corresponding to values of D. How these are obtained will now be considered.

Viability in continuous culture

The specific death rate can be conveniently calculated

from equation (2) which will give :

$$\mu_d = D(x^T/x^V - 1)$$

x^T/x^V is the viability of the population in terms of biomass. However, the viability (V) is actually measured in terms of cell numbers so it is necessary to make the assumption that there is no difference in mass between viable and "non-viable" cells. This may not be justified for Errington, Powell and Thompson (1965) found that "non-viable" cells differ in size from viable cells. Also if "non-viable"cells "grow" then this relationship will be affected. However, providing the viability of the population is high or that any difference in mass is small, the error involved in using V will be small. We then have :

$$\mu_d = D(1/V - 1)$$

or:

$$\mu = D + \mu_d = D/V \qquad (13)$$

Equation (13) is not the same as an expression given elsewhere for the growth rate in cultures where part of the population is non-viable (Tempest, Herbert and Phipps, 1967; Tempest, 1970; but see Pirt, 1975). This expression, with the growth rate denoted by μ^*, is :

$$\mu^* = D \ln 2/\ln 2\alpha$$

where α is the index of viability. Since :

$$\alpha = (V + 1)/2$$

(Powell, 1956):

$$\mu^* = D \ln 2/\ln (V + 1)$$

which is only the same as the expression for μ when $V = 1$. The difference is due to the fact that μ^* is defined from :

$$\mu^* = \ln 2/g$$

whereas μ is defined from :

$$dx^T/dt = \mu x^V \qquad (14)$$

g is called the doubling time of viable cells but this is misleading since during this time the numbers of viable cells do not actually double as part of the progeny are non-viable. In fact over g each part of the population, viable, non-viable and total, increases by a factor of 2α. g has been called the mean generation time but this again is incorrect as g does not correspond to the mean of the distribution of individual generation times (Powell, 1956; Painter and Marr, 1967).

Another consequence of the population increasing by 2α is that no equivalent to equation (14) can be given for μ^* and its precise meaning is obscure. μ is a more practical measure of the growth rate, particularly as it bears a direct relationship to q^V, the rate of consumption of substrate by the viable biomass. The relationship between μ and μ^* is given by :

$$\mu^* = \mu V \ln 2/\ln (V + 1)$$

No model of the kinetics of death in continuous

culture has so far been postulated to describe how V varies with D. An arbitrary formula has been suggested by Powell (1967) which will mathematically describe the observed shape of the curve of V in the few experimental studies undertaken:

$$V = \mu/(\mu + \mu_v)$$

where μ_v is a constant. This indicates that V is practically unity at high values of μ but then decreases with μ at low values of μ. It would seem preferable to incorporate a value called V_{max} since V at high growth rates is not necessarily unity as has been indicated in Arthrobacter globiformis (Luscombe, 1972). Also it is clear that when Powell uses μ he has in mind D since μ, as defined in this study, is not an independant variable but itself varies with V. The revised model is :

$$V = V_{max}\, D/(D + V_{max}\, \mu_{min}) \qquad (15)$$

where μ_{min} is another constant.

A useful feature of this model is that it incorporates the idea of a minimum growth rate. It has been suggested that microorganisms can only grow at a certain minimum rate and if nutrient is supplied at a rate lower than this, then part of the population dies or enters a resting state. Strong evidence for a minimum growth rate has been given for Klebsiella pneumoniae (Tempest et al. 1967) and the existing position has been

reviewed by Pirt (1972). Substituting (13) into (15):

$$D/\mu = V_{max}D/(D + V_{max}/\mu_{min})$$

$$\mu = D/V_{max} + \mu_{min} \qquad (16)$$

From this it can be seen that when D tends to zero, μ tends to μ_{min}.

If a minimum growth rate really does exist, at least for some microorganisms, then if equation (9) is considered it is seen that as D tends to zero the yield will not tend to zero but to a minimum value. Of course, as soon as D is zero the viability is zero and a steady state is no longer possible. However it is probable that before such a state is reached and when a large proportion of the population is "non-viable", other factors such as cell leakage will complicate the situation. With equations (11) and (12) no minimum yield is suggested.

Correction for evaporation

King, Sinclair and Topiwala (1972) have pointed out the need to correct measurements of maintenance energy taken in continuous culture for errors due to evaporation losses. They show that the specific maintenance rate is underestimated by an amount D_2, the effective evaporation rate given by :

$$D_2 = F_2/v$$

where F_2 is the evaporation rate of water into the air stream. This equation can be applied directly to

equations (11) and (12) but results in a slight under-correction when applied to equation (9). The evaporation rate has no effect on the value of Y_{max}.

METHODS

Apparatus

The chemostat used in these studies was a Biotec 3l fermentor (LP100 instrumentation and FL103 vessel) fitted with a gas outlet condenser. The impeller was maintained at 500 r.p.m. No foam control was required. The hydrogen ion concentration was steady during cultivation at pH 6.7 $\pm$ 0.1 and, since the medium was buffered, no control was required. The temperature was maintained at 25°C; the coolant for the cold finger and the gas outlet condenser was provided by the cold water supply which was adequate in all but the hottest weather. The air flow was 2.30 l min^{-1}, a small fraction of which was diverted through a one-tier dropper system on the medium feed line similar to that described by Luscombe (1972) to prevent back-contamination. The dissolved oxygen concentration was steady at 37% saturation. The chemostat vessel was shielded from direct daylight.

A 50ml burette was put onto a T-junction on the medium line to facilitate accurate measurement of the medium flow rate. A double line of silicone tubing provided a standby for the peristaltic pump. A T-junction on the culture overflow line facilitated collection

either in a 1.3l replaceable sample bottle or a 20l receiving vessel. Connections between the medium vessel, chemostat and receiving vessel were made via Portex autoclavable male/female junctions and the three units were autoclaved separately.

The working volume of the chemostat under the specified conditions was found to be 2665 (s.e.) 8ml. A small number of studies were carried out using an all-glass, magnetically stirred chemostat as described by Luscombe (1972) of 140.9ml working volume, although the input air was sparged through water to reduce evaporation losses. Two runs at high dilution rates were done in a FL10l vessel with the impeller at 650r.p.m. giving a working volume of 1031 (s.e.) 5ml.

Media

The medium used was the mineral salts medium base E of Owens and Keddie (1969) with added biotin at 1 $\mu g l^{-1}$ (Chan and Stevenson, 1962) and made up in deionized water. The medium was autoclaved in 20l vessels for either 40min in a Baird and Tatlock vertical autoclave or 75min in a Drayton-Castle wheel-in autoclave. The loss of volume during autoclaving was measured for each batch of medium and a correction applied to the concentration of the limiting substrate. A slight decrease in pH was noted after autoclaving. A heavy precipitate formed during autoclaving but this redissolved

on cooling; the trace remaining settled out and did not pass into the chemostat.

The medium designed for carbon limitation contained the standard amount of ammonium sulphate (0.5 g l^{-1}) and glucose at 0.2g l^{-1}. That carbon was limiting was shown by the increase in absorbance by a chemostat culture in equilibrium on further addition of glucose (see p. 53). The medium for nitrogen limitation contained ammonium sulphate at 0.05g l^{-1} and glucose at 0.7g l^{-1}. That nitrogen was limiting was similarly shown by an increase in absorbance with the addition of ammonium sulphate but no increase with the addition of glucose. In all cases the glucose was autoclaved separately except at 0.2g l^{-1} in the vertical autoclave otherwise some caramelization was observed.

Growth of cultures.

The organism used in these studies was obtained initially from a freeze-dried culture of Arthrobacter globiformis NCIB 10683. This was maintained in stock cultures on nutrient agar slopes stored at 4-10°C under oil. The inoculum for the chemostat was prepared by growing the organism in peptone-yeast extract broth for at least 24h and injecting 2ml through the inoculation port. The chemostat was never inoculated with a culture that had been previously cultivated continuously. The culture was allowed to grow under batch conditions for at least

24h. The culture was considered to be in a steady state after 5 volumes of medium had passed through the chemostat although at the high flow rates more than this had usually passed through before any sampling was undertaken.

Samples of cells for most analyses were collected in the 1.3l bottle surrounded by an ice jacket. Larger quantities were similarly collected in the 20l vessel and the cells harvested using an Alfa-Laval LAB 102 B-20 separator followed by freeze-drying (Speedivac, Edwards High Vacuum).

Cell Weight determinations

The cell concentration was measured by weighing the cells on membrane filters. A known volume, 50-300ml, of chilled chemostat overflow was pipetted into a Millipore Sterifil apparatus containing a cellulose nitrate membrane filter, pore size 0.45 m (Sartorius, Gottingen, West Germany, catalog no. 11306) which had been washed in deionized water, dried and preweighed. After filtration ca. 10ml cold deionized water was flushed through to remove adhering medium. The filter was predried overnight at 4-10°C in a desiccator containing silica gel and then dried *in vacuo* at 45°C after Sartory, Merer and Buri (1947) for three hours. The predrying in the cold prevented the cells loosing weight by respiration during drying. Drying was attempted at 105°C but this produced very brittle filters which

broke up on handling and which stuck to the glass petri dishes in which they were dried. After three hours drying *in vacuo* any further change in weight was only 0.1mg, the limit of the balance used. Further drying at 105°C showed only ca. 0.1mg loss. It was found necessary to have a desiccated balance as the cells rapidly absorbed moisture. By this method 8-30mg cells were weighed on filters of ca. 100mg.

Viability measurements

Viability was measured using a modification of the slide culture technique of Postgate, Crumpton and Hunter (1961). The medium was that used by Luscombe (1972) and was filtered to remove contaminating bacteria which could otherwise be erroneously counted as dead cells. This was poured into disposable petri dishes (Sterilin) to a depth of 1.0mm from which a large number of $1cm^2$ blocks could be cut. These were placed on sterile microscope slides in a moist chamber. Cells were taken directly from the chemostat overflow and diluted ca. 50X into peptone-yeast extract broth from which a loopful was transferred to the surface of an agar block. Without allowing the inoculum to dry a sterile coverslip was placed on top of the block and the moist chamber incubated at 25°C. Cells from growing populations usually gave countable colonies within 24h. At least 300 objects were counted for each determination (Postgate, 1967) usually

on three slides using phase-contrast microscopy (Zeiss, Standard RA Routine and Research microscope) under oil immersion at 800X. The viability was calculated from the grand total of cells counted and the standard deviation was calculated from the square root of this total as counts made in this manner should have a Poisson distribution. Counts on individual slides were observed to fall within this deviation. Occasionally some slides were observed with much lower viabilities; these usually took longer to develop or showed a large variation in viability on the one slide. They were attributed to toxicity of unknown origin and were ignored in the calculations.

RESULTS AND DISCUSSION

Viability

Figure 1 shows the viability measurements for a carbon-limited population. A slight decrease is shown from high to low dilution rate but the values average about 90%. This is in agreement with Luscombe (1972) who pointed out their dissimilarity from measurements in other bacteria such as Klebsiella pneumoniae which show almost 100% viability towards wash-out but a much greater drop-off at low dilution rates.

An attempt was made to fit the data to equation (14) by using a double reciprocal plot :

$$1/V = 1/V_{max} + \mu_{min}/D$$

However in the linear regression analysis the correlation coefficient was not significant. The fitted curve in figure 1 corresponds to the best eye-fit line to the error bars on the double reciprocal plot but the fit is poor particularly at the lowest dilution rate. The parameters are :

$$V_{max} = 0.924$$

$$\mu_{min} = 0.0010 \quad h^{-1}$$

More accurate viability data could show μ_{min} to be lower than this.

Equation (14) was also applied to the data points of Tempest et al. (1967) for K. pneumoniae and a good fit was obtained (correlation coefficient, $r = 0.9977$; significant at $p < 0.001$) indicating that the model described by (14) could be useful and meaningful. The parameter values are :

$$V_{max} = 1.043 \text{ (s.e) } 0.024$$

$$\mu_{min} = 0.0070 \text{ (s.e) } 0.0002 \quad h^{-1}$$

The value for μ_{min} is close to that estimated by Tempest et al. at 0.009 h^{-1}. The value of V_{max} cannot actually be more than one. The indication is that the value of μ_{min} for A. globiformis is at least seven times smaller than that of K. pneumoniae although it should be remembered that the data for the latter was obtained at 37°C. The authors report an increased viability in their cultures at 25°C which would give a lower minimum growth rate.

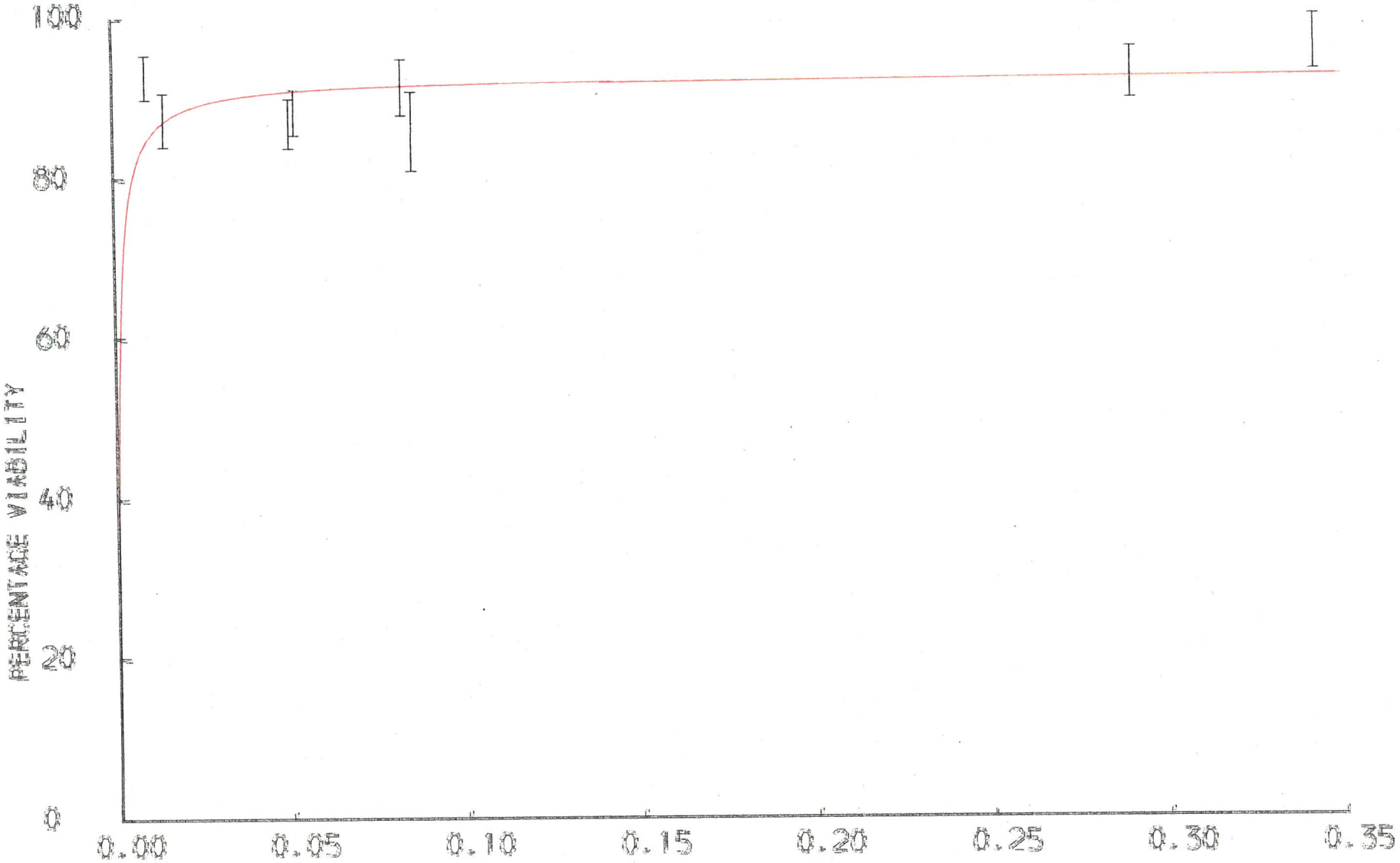


FIGURE 1 EFFECT OF DILUTION RATE ON VIABILITY OF CARBON-
-LIMITED CELLS. BARS REPRESENT STANDARD DEVIATIONS. RED
LINE SHOWS FITTED CURVE.

Pirt (1972) pointed out the need for caution in interpreting viability measurements made by plating out on rich media and this may be relevant to the data of Tempest et al. in that some of the cells counted as viable would not have been able to divide in the chemostat environment but were present in some sort of dormant state. One could argue the reverse in that bacteria which would have grown in the chemostat could be rendered non-viable by the metabolic shock of a sudden nutrient-rich environment. Luscombe (1972) however showed that there was no significant differences between the viabilities of A. globiformis as measured on rich and minimal media for both dilution rate = 0.01 h^{-1} and 0.30 h^{-1}.

Cell Yield

The data points for the cell yield are shown in figure 2. The low values at D = 0.31 h^{-1} were obtained in the 1l fermentor from a population on the point of washout. The reason for this is not known; if the population is nearly 100% viable then washout should not occur until D approaches μ_{max} (ca. 0.40 h^{-1}). The viability on this run was not determined. A double reciprocal plot of cell yield and dilution rate is shown in figure 3. The values can be seen to lie on a straight line up to a mean retention time of 38h corresponding to D = 0.026 h^{-1}. At lower values of D a sharp departure occurs where the yield actually increases.

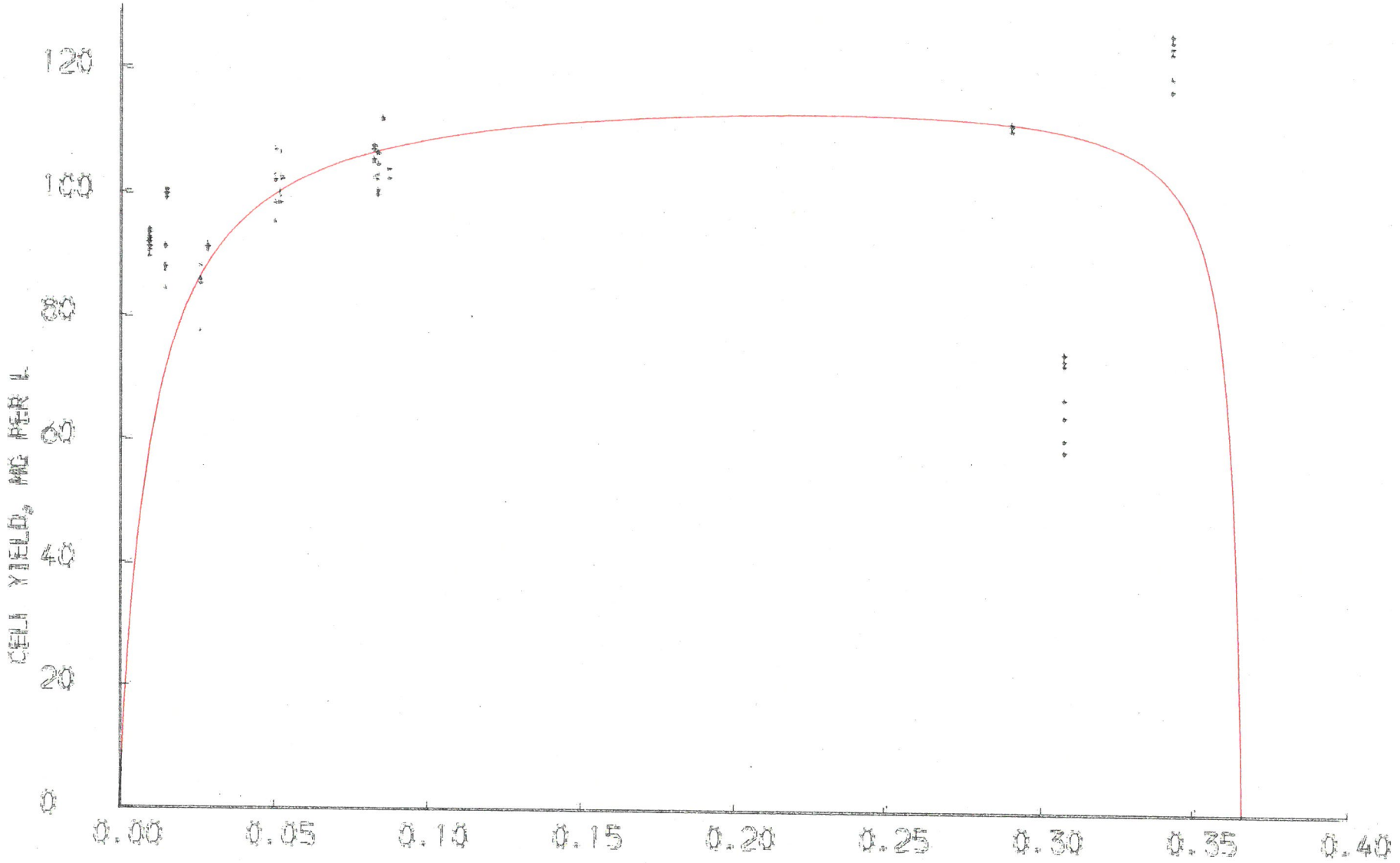


FIGURE 2. EFFECT OF DILUTION RATE ON CELL YIELD OF CARBON-LIMITED CELLS WITH GLUCOSE AT 200.0 MG PER L. RED LINE SHOWS FITTED CURVE.

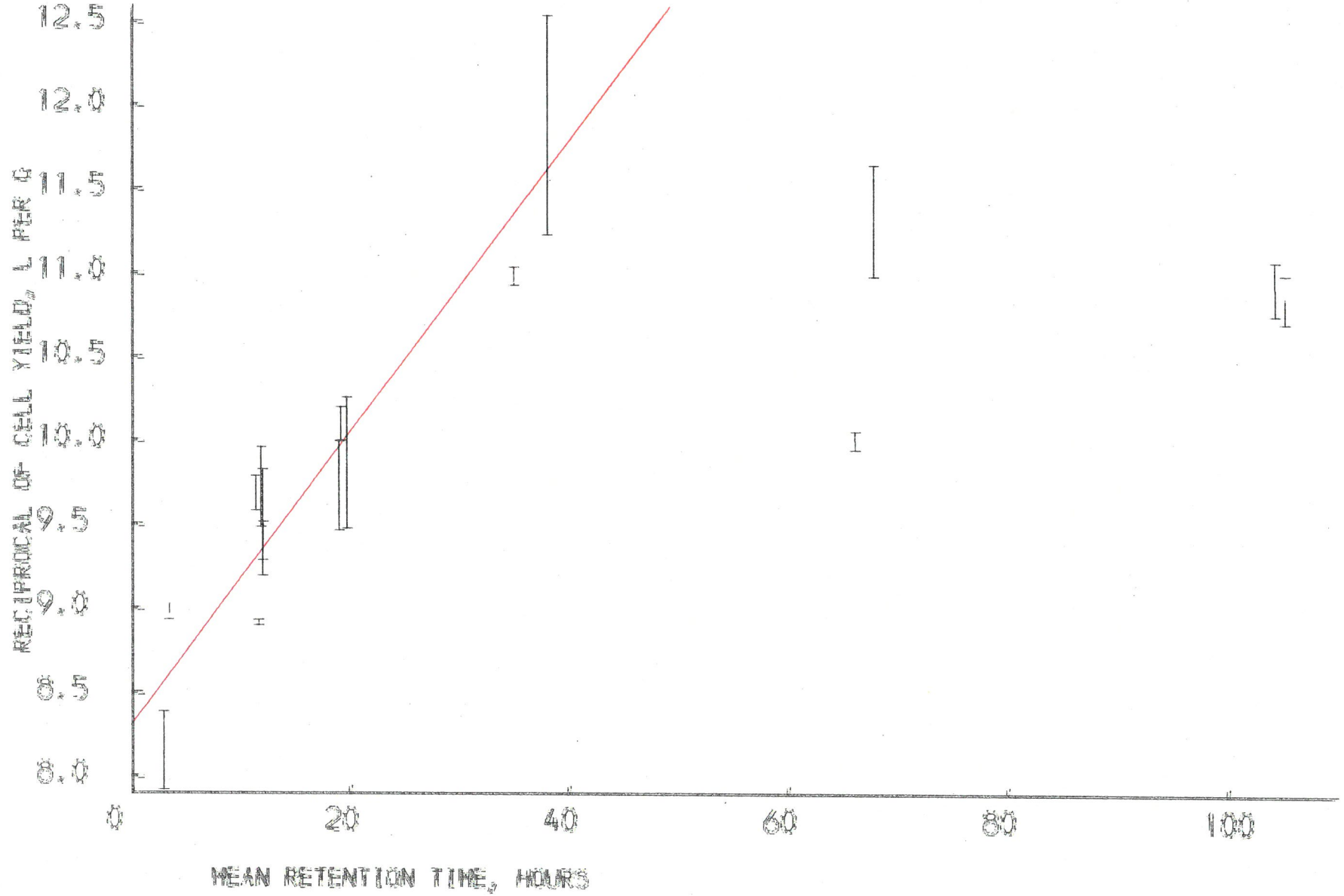


FIGURE 3 RECIPROCAL PLOT OF DILUTION RATE AND CELL YIELD OF CARBON-LIMITED CELLS WITH GLUCOSE AT 200.0 MG PER L. RED LINE IS ACCORDING TO PARAMETERS GIVEN BY LINEAR REGRESSION ANALYSIS ON DATA POINTS UP TO A MEAN RETENTION TIME OF 38 HOURS.

The plot in figure 3 is the test for equation (11) and the fitted line was obtained by linear regression analysis on the data up to 38 h (excluding the washout values from the 11 fermentor). The parameters of the regression line are given in table 4 along with those obtained by fitting the same data to equations (9) and (12). With the latter two equations it was necessary to determine the values of μ from the viability and the dilution rate. These were calculated using equation (13), the viabilities being given from the fitted curve in figure 1. The correlation coefficients for the regression lines of the three equations were all highly significant ($p < 0.001$). The fitted curve in figure 2 was obtained from equations (11) and (16) with the parameters :

Y_{max}	=	0.6017	g g^{-1}
S_r	=	0.200	g l^{-1}
K_s	=	0.002	g l^{-1}
V_{max}	=	0.9244	
μ_{min}	=	0.0010	h^{-1}
μ_e	=	0.01046	h^{-1}
μ_{max}	=	0.40	h^{-1}

The differences in the values of the parameters given in table 4 reflect the nature of the assumptions behind the equations. Equation (9) gives a higher value for μ_e than equation (11) since all the decrease in yield is attributed to the maintenance of viable cells. In

Table 4. Maintenance parameters and growth yield of Arthrobacter globiformis obtained by fitting cell yield data (46 data points) by linear regression analysis to the three selected equations.

			Equation		
			(9)	(11)	(12)
Maximum yield factor	Y_{max}	g g^{-1}	0.6033	0.6017	0.6510
(s.e.)			0.0082	0.0081	0.0087
Maintenance coefficient	q_e^v	g g^{-1} h^{-1}	0.01949	0.01738	0.01590
(s.e.)			0.00126	0.00113	0.00115
Specific maintenance rate	μ_e	h^{-1}	0.01176	0.01046	0.01035
Determination coeff.	r^2		0.8444	0.8441	0.8134
Specific maintenance rate (corrected)	μ_e	h^{-1}	0.01212	0.01082	0.01071

equation (11) the hypothesis is that both the viable and the non-viable cells are being maintained. The values given will be unaltered even if the "non-viable" cells "grow". Equation (12) gives a similar value for μ_e to equation (11) since again both viable and"non-viable" cells are being maintained. However the value of Y_{max} is markedly increased because of the assumption that the substrate is being wasted by the "non-viable" cells giving much lower yields than would otherwise be theoretically possible.

As has been mentioned it is not possible to say which model is the correct one and so determine which equation gives the true parameters. Perhaps the most likely situation is where the "non-viable" population requires some maintenance but not as much as the viable population which would give a value for μ_e intermediate between that given by equation (9) and those given by equations (11) and (12); and also that a degree of uncoupling occurs giving a value of Y_{max} intermediate between that given by equation (12) and those given by equations (9) and (11). The goodness of fit of the data to the models cannot give any indication to their correctness either since although the values of r^2 are slightly higher for equations (9) and (11) than for equation (12), the differences are not statistically significant.

Significance of the growth yield

The growth yield of an organism is important in giving an indication of the efficiency with which it utilizes its energy source. If the limiting substrate is both the carbon and the energy source then the distribution between the two has to be taken into account. If it is assumed that all the carbon not in the cells is oxidised with equal efficiency to carbon dioxide the values for the molar growth yield, Y_{glu}, given in table 5 are obtained. Y_{glu} is the yield of cells per mole of glucose used as energy source. The calculation method is given in appendix III .

Table 5. Molar growth yield and Y_{ATP} of *Arthrobacter globiformis* NCIB 10683

Equation		(9)	(11)	(12)
Y_{glu} (all non-cellular carbon oxidised)	g mole^{-1}	290.0	288.0	360.5
Y_{ATP}	g mole^{-1}	7.83	7.78	9.73
Y_{glu} (material balance method)	g mole^{-1}	308.5	306.1	389.5
Y_{ATP}	g mole^{-1}	8.33	8.26	10.52

An alternative approach is to consider the material balance where, since the composition of bacterial cells usually differs from that of the energy and carbon source, it is found that not all the carbon respired as carbon

dioxide requires oxidation. Chen (1964) used this method with studies on Torulopsis utilis and the calculation method is given in appendix III.

By considering the yield of moles of ATP per mole of glucose oxidized by various metabolic pathways it is possible to calculate the Y_{ATP} values given in table 5. The Embden-Meyerhof pathway (EMP) yields 38 moles of ATP per mole of glucose oxidized whereas the pentose phosphate cycle or hexose monophosphate pathway (HMP) yields 35 moles of ATP per mole of glucose oxidized. Zagallo and Wang (1962) showed that in their strain of Arthrobacter globiformis, 68% of the glucose was oxidized via the EMP and 32% via the HMP. Morris (1960) made a similar finding. On this basis a mean of 37.04 moles of ATP produced per mole of glucose can be calculated. It has been suggested that Y_{ATP} is a biological constant and several studies have shown it to be about 10.5 (Senez, 1962; Stouthamer, 1969; Payne, 1970). The values in table 5 are lower than this apart from that given by equation (12) and the material balance method. From this it could be tentatively suggested that equation (12) represents the best model although Stouthamer and Bettenhausen (1973) have shown that Y_{ATP} values may cover a wide range and that it may not be the biological constant once thought. The value of Y_{ATP} will also vary with the nature of the medium, higher values being obtained in complex media where performed units of amino acids,

etc. are available. Correcting the values in table 5 for the energetic demand of monomer synthesis would increase them by about 6% (Senez, 1962).

Correction for evaporation rate

Although the chemostat was fitted with an air condenser it was thought that the evaporation rate could be significant particularly as the air supply was known to be very dry. The outflowing air was passed through a pre-weighed U-tube packed with anhydrous copper sulphate. The weight increased by 1.95g over 2.02 h which gives an effective dilution rate due to evaporation of 0.000362 h^{-1}. About 7% of the air flow exits via the waste culture pipe but when collecting cells at 0°C, the water vapour in this stream will be condensed. The corrected values for μ_e are given in table 4.

Maintenance energy at low growth rates

The significance of the cell yields at low growth rates will be considered. Figure 3 shows that above a mean retention time of 38 hours, the cell yields obtained are higher than expected. This means that the maintenance requirement must no longer be constant and independant of the growth rate but that it must have decreased. It is possible to calculate the specific maintenance rate using the model equations and assuming Y_{max} to be constant throughout.

From equation (11) we obtain:

$$\mu_e = D(Y_{max}/Y - 1)$$

The values for μ_e so calculated are plotted in figure 4 against the mean retention time. The values similarly obtained for Klebsiella pneumoniae from the data of Tempest et al. (1967) are also given for comparison. The large scatter of points at small mean retention times is to be expected since the values depend on the small difference between Y_{max} and Y. The graphs could be drawn using equations (9) or (12). These make little difference to the magnitude of μ_e for A. globiformis but due to the low viability of the K. pneumoniae population, μ_e is changed much more. Equation (9) gives higher values for μ_e and equation (12) lower values. Whichever model is used, the specific maintenance rate of A. globiformis is always about four times lower than that of K. pneumoniae at the corresponding mean retention time.

Some possible mechanisms by which the maintenance requirement can be reduced have been discussed earlier (p.7). These might apply to the whole of the population or alternatively the population may be heterogeneous such that part requires maintenance at the normal rate whilst the remainder has a "shut-down" metabolism but is nevertheless capable of division when the nutrient status becomes favourable. Thus the measured maintenance requirement would be the mean of these two parts. Evidence for heterogeneity has been given for Escherichia coli at low dilution rates (Koch and Coffman, 1970) where the two parts

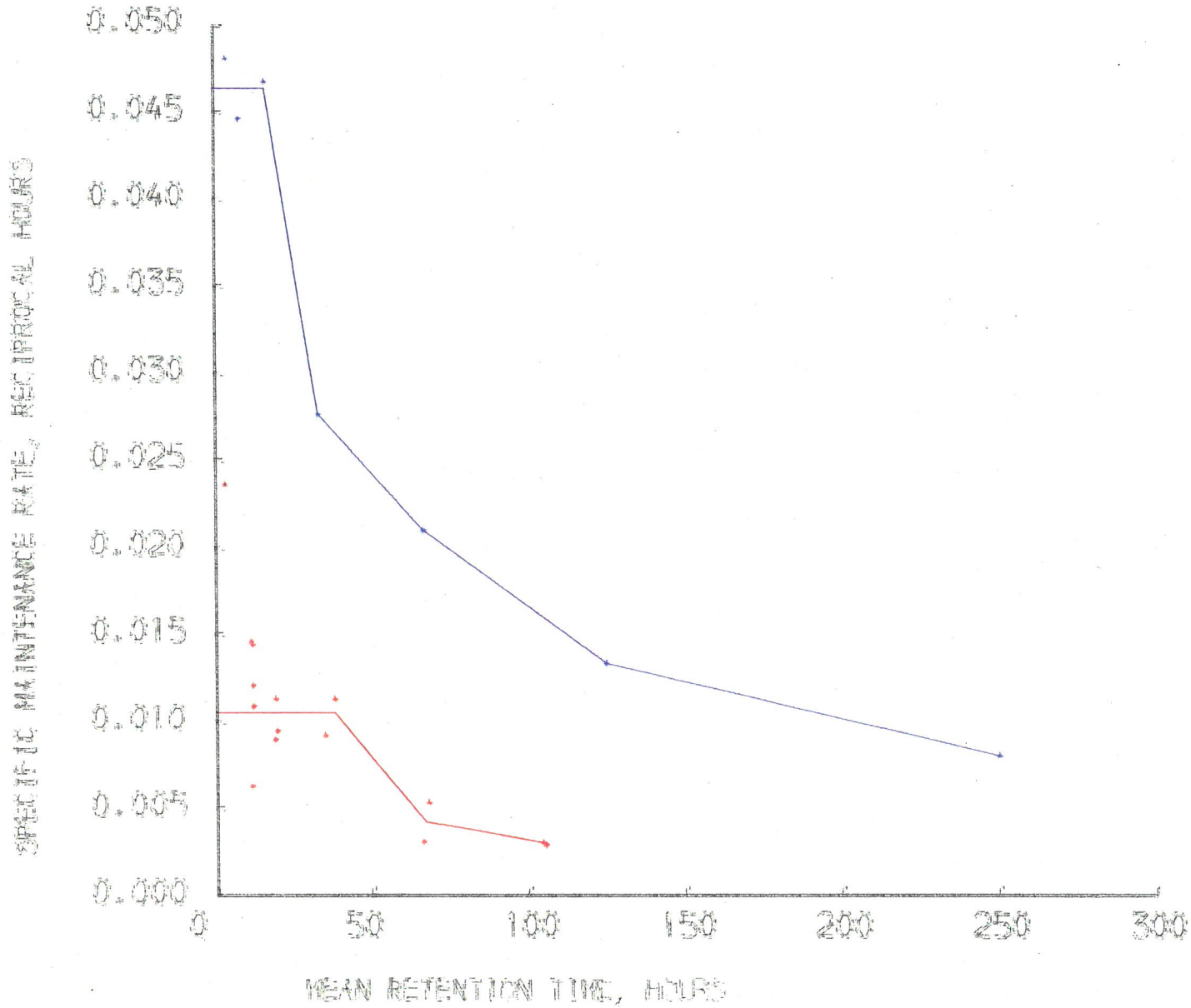


FIGURE 4. GRAPH SHOWING DECREASE IN SPECIFIC MAINTENANCE RATE AT LOW DILUTION RATES USING EQUATION (1). RED, *AEROBACTER AEROGENES* BLUE, *KLEBSIELLA PNEUMONIAE*

of the population differed in their abilities to induce β-galactosidase. Such heterogeneity is also suggested in the viability measurements of Arthrobacter globiformis where a scatter of division lags have often been observed although no quantitative measurements were made.

Change of maintenance energy with temperature

Measurements of cell yield and viability were made at a range of temperatures using the 140ml chemostat, the results being given in table 6. Due to differences in the glucose concentration in the media the results have been corrected to make them comparable with those obtained in the 3l fermentor. Experience with this design of chemostat has shown that the culture volume can vary considerably with the speed of the magnetic stirrer which in turn can vary during a run. The dilution rates given should therefore be treated as approximate. The values at 25°C, given for comparison are calculated from the fitted lines in figures 1 and 3.

Viability. It can be seen that for one particular temperature the viability decreases as the dilution rate decreases and that for any one dilution rate the viability shows no significant change. Tempest et al. (1967) reported a large increase in the viability of their chemostat population of K. pneumoniae when the temperature was lowered from 37 to 25°C but in their case the viabilities were much lower than those given by A. globiformis.

Table 6. Effect of temperature on the viability and specific maintenance rate of Arthrobacter globiformis.

Dilution rate h^{-1}			Temperature			
			10 °C	15 °C	20 °C	25 °C
0.0125	Cell yield	mg l^{-1}		92.9 (s.d. 5.7)		
	Viability	%		88.5 (s.d. 3.6)		85.6
	Specific maintenance rate	h^{-1}		0.00369		
0.019	Cell yield	mg l^{-1}	108.1 (s.d. 1.1)			
	Viability	%	82.2 (s.d. 8.8)			87.8
	Specific maintenance rate	h^{-1}	0.00215			
0.0516	Cell yield	mg l^{-1}	Washout	112.9 (s.d. 3.0)	106.0 (s.d. 2.0)	100.0
	Viability	%		88.9 (s.d. 2.9)	88.9 (s.d. 2.3)	90.6
	Specific maintenance rate	h^{-1}		0.00342	0.00696	0.01046
0.104	Cell yield	mg l^{-1}	Washout	116.4 (s.d. 2.0)	114.8 (s.d. 2.5)	109.3
	Viability	%		94.7 (s.d. 4.2)	91.8 (s.d. 5.4)	91.5
	Specific maintenance rate	h^{-1}		0.00356	0.00505	0.01046

Maintenance energy. The cell yields decrease with decreasing dilution rate and with increasing temperature. This suggests that the maintenance requirement increases with temperature. The data was considered to be too scanty to justify making double reciprocal plots so it was assumed that the maximum yield was invariant with temperature and approximate values for the specific maintenance rate calculated based on equation (11). These values are given in table 6. The calculations could also have been made based on equations (9) and (12). The specific maintenance rate values are approximately the same at the same temperature but decrease appreciably with decreasing temperature.

If the logarithms of the specific maintenance rates are plotted against the reciprocals of the absolute temperatures, i.e. an Arrhenius plot, a straight line is obtained (correlation coefficient is significant, $p = 0.0008$). From the slope of the line the activation energy of the maintenance process can be obtained following the form of the Arrhenius equation:

$$\ln K = \ln A - E/RT$$

where K is the rate of the process, A is a constant, E is the activation energy, R is the universal gas constant and T is the absolute temperature. Hence E = 74.1 kJ mole $^{-1}$. This is comparable to the value of E for E. coli strain PS at 83.4 kJ mole^{-1} (Marr et al. 1963) and is slightly less than the value of E for the growth rate at approximately 91.6 kJ mole^{-1} (Chapman, 1972,

unpublished data). The relatively high value of E for maintenance suggests that maintenance is more involved in providing energy for metabolic processes than for diffusion processes as the activation energies of diffusion are smaller than those of metabolism. Mennett and Nakayama (1971) came to this conclusion on finding that in Pseudomonas fluorescens E for growth, 69.5 kJ $mole^{-1}$, was very similar to E for maintenance, 76.4 kJ $mole^{-1}$. Palumbo and Witter (1969) found a lower value of E for maintenance in a psychrophilic strain of Ps. fluorescens at 35.3 kJ $mole^{-1}$. This might be expected as values of E for growth are lower for psychrophiles than for mesophiles.

MEASUREMENT OF SATURATION CONSTANT

Introduction

Monod's (1950) relationship between substrate concentration and consumption, equation (6) :

$$q^v = q^v_{max}\ s/(K_s + s)$$

contains the value K_s known as the saturation constant. It may be considered as representing the affinity of a microorganism for a particular substrate; the smaller the value of K_s, the greater the affinity. Alternatively it represents the ability of a microorganism to grow at an appreciable rate even when its limiting substrate becomes reduced to low concentrations. The value of K_s has considerable ecological importance from the point of view

of an organism scavenging nutrients in low concentrations from its environment and also as a factor in determining the outcome of competition between two or more organisms for one substrate. The outcome of such competition depends on the relative growth rates of the species concerned which in turn depends on the maximum growth rates, the values of K_s and the prevailing substrate concentration (Veldkamp and Jannasch, 1972; Meers, 1973). This treatment does not take into account the effect of endogenous metabolism which could be important at very low growth rates where a considerable proportion of the substrate taken into an organism is diverted to maintenance. Given two competing bacteria with identical values of q^v under a certain set of conditions, the one with the lower maintenance requirement will out-compete the other since more substrate will be available for growth.

From the above it was considered useful to obtain the value of K_s of Arthrobacter globiformis NCIB 10683 for glucose. Estimations of the value have been made by two approaches.

Experiments following glucose uptake

One way of measuring K_s is by observing substrate uptake over the region where its concentration limits growth and seeing how the growth rate decreases as the substrate

concentration tends to zero. The method used was adapted from that used by Koch (1971) for determining K_s in Escherichia coli.

Method. Cells of Arthrobacter globiformis were centrifuged from 50ml. culture taken from the 140ml chemostat operated at a dilution rate of 0.3 h^{-1} and with glucose as the limiting substrate at 0.2 g l^{-1}. These were resuspended in 49ml of mineral base E plus biotin and placed in a 100ml. boiling tube held in a water bath at 25°C. The culture was mixed and aerated by sparging with air. The absorbance was measured by cycling the culture at 400ml h^{-1} through the continuous flow cell of a UVicord II 8300 U.V.-absorbtiometer (LKB, Sweden) operated at 254nm. It would be preferable to use a wavelength nearer 420nm since U.V. light is much more prone to errors due to scattering than visible light. However a series of tests using diluted suspensions of bacteria showed that the absorbance was proportional to cell concentration within the range used in these experiments. A change in cell shape or size can also have a marked effect on the absorbance but these experiments used rapidly-growing rods throughout so that any errors from this cause are negligible. 1ml of a solution of glucose was added to the culture and the subsequent growth was continuously traced using a Kipp and Zonen BD 8 flatbed recorder. The absorbance of the initial culture was

measured against water and then the absorbance was reset to zero so that the absorbance measurements then reflected the growth on the added substrate. In view of the time periods involved sterile precautions were not necessary.

Results. These are shown in figures 5-8 where different concentrations of glucose have been added except for figure 8 where sucrose was added. The recorder trace gave the results in terms of transmission values so it was necessary to convert these to absorbance values to obtain the curves drawn. These all show an exponential increase, an abrupt cut-off and then a very gradual increase in absorbance. The exponential nature of the curve is readily seen in figure 5 where the logarithm of the total absorbance has also been plotted and which is practically linear with time. The total absorbance is the sum of the absorbance of the initial culture (0.1342) and the extra absorbance due to growth on the added glucose. The slope of this line will give the specific growth rate which was found to be 0.254 h^{-1}. This is less than the maximum specific growth rate which is about 0.4 h^{-1} suggesting that the cells have not fully recovered from the centrifuging and resuspending process and/or that the population is partly non-viable.

The exponential nature of the curves is less obvious in figures 6-8 and is due to the smaller quantities of

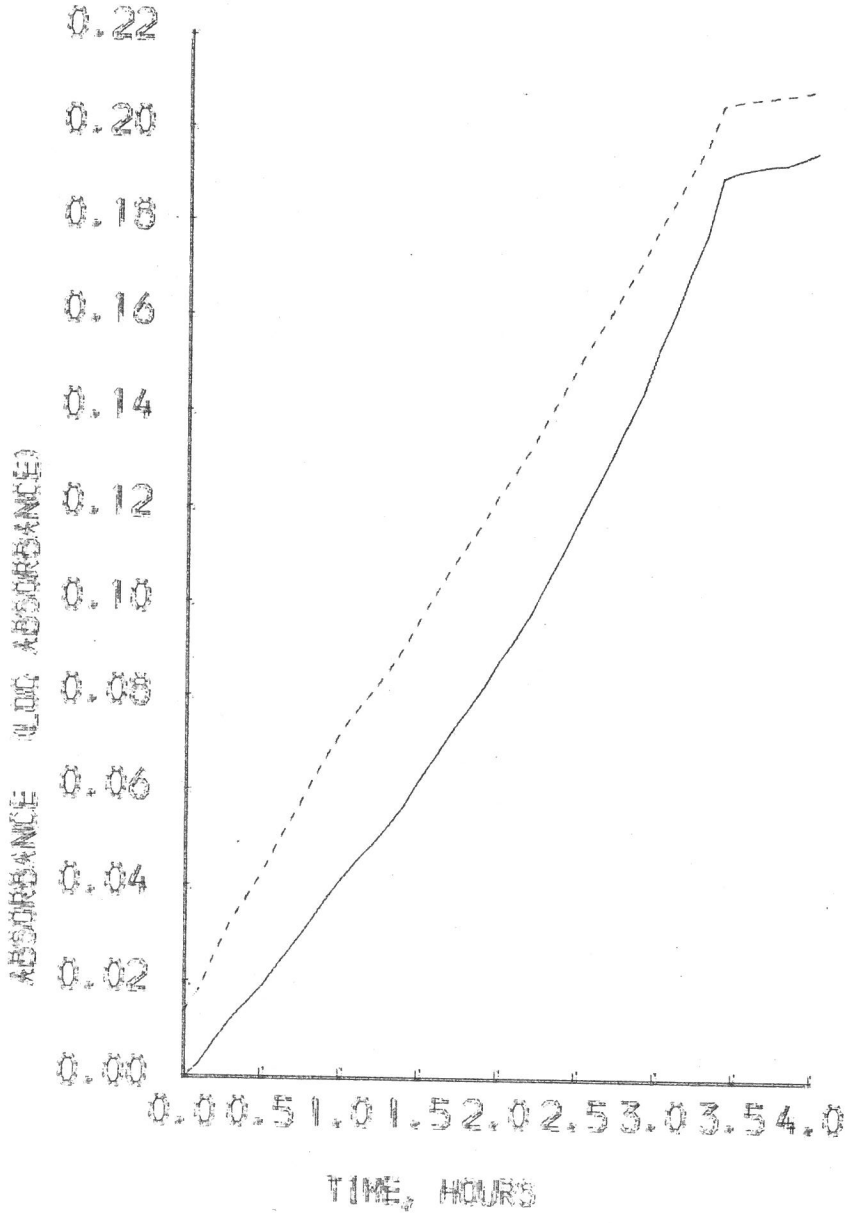


FIGURE 5 GROWTH OF ARTHROBACTER GLOBIFORMIS WITH 200 MG PER L GLUCOSE DASHED LINE SHOWS LOGARITHMIC PLOT (ANNOTATION NOT GIVEN)

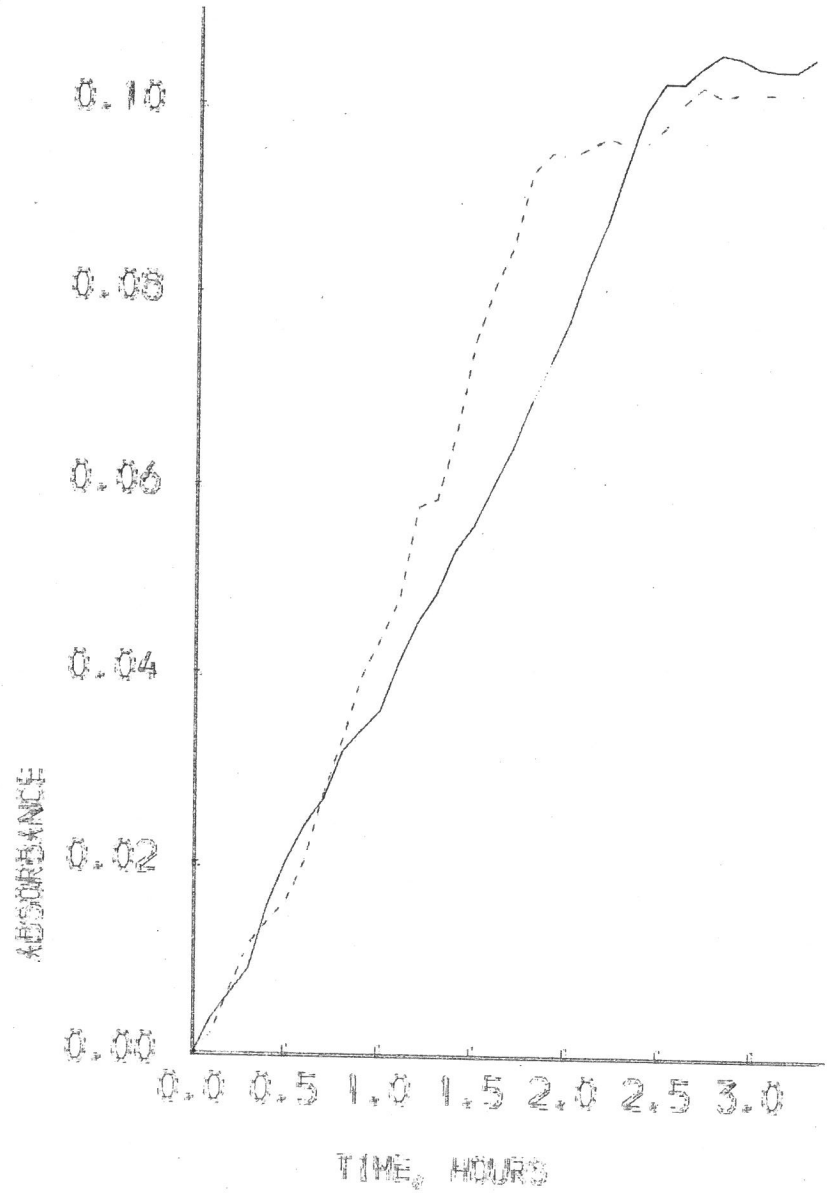


FIGURE 6 GROWTH WITH 100 MG PER L GLUCOSE. TWO REPLICATES.

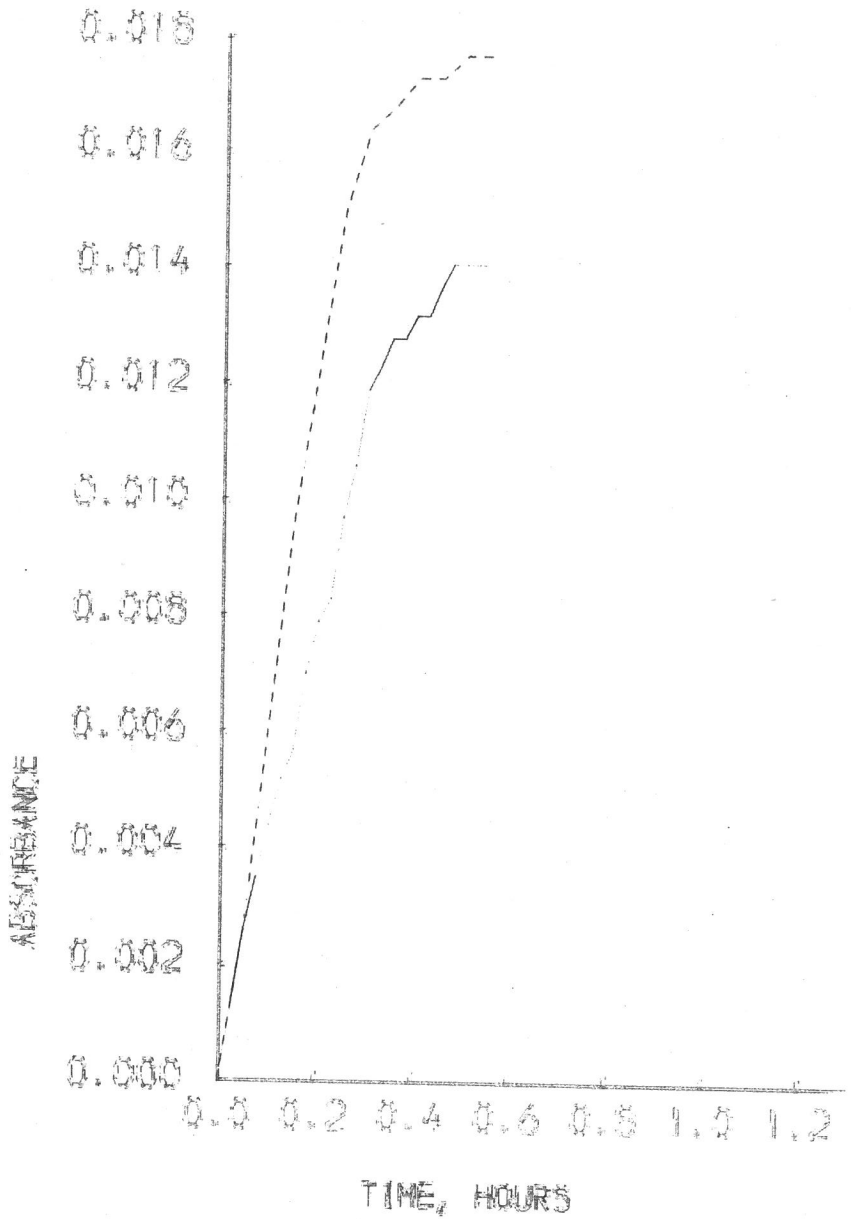


FIGURE 7 GROWTH OF ARTHROBACTER GLOBIFORMIS WITH 20 MG PER L GLUCOSE. TWO REPLICATES.

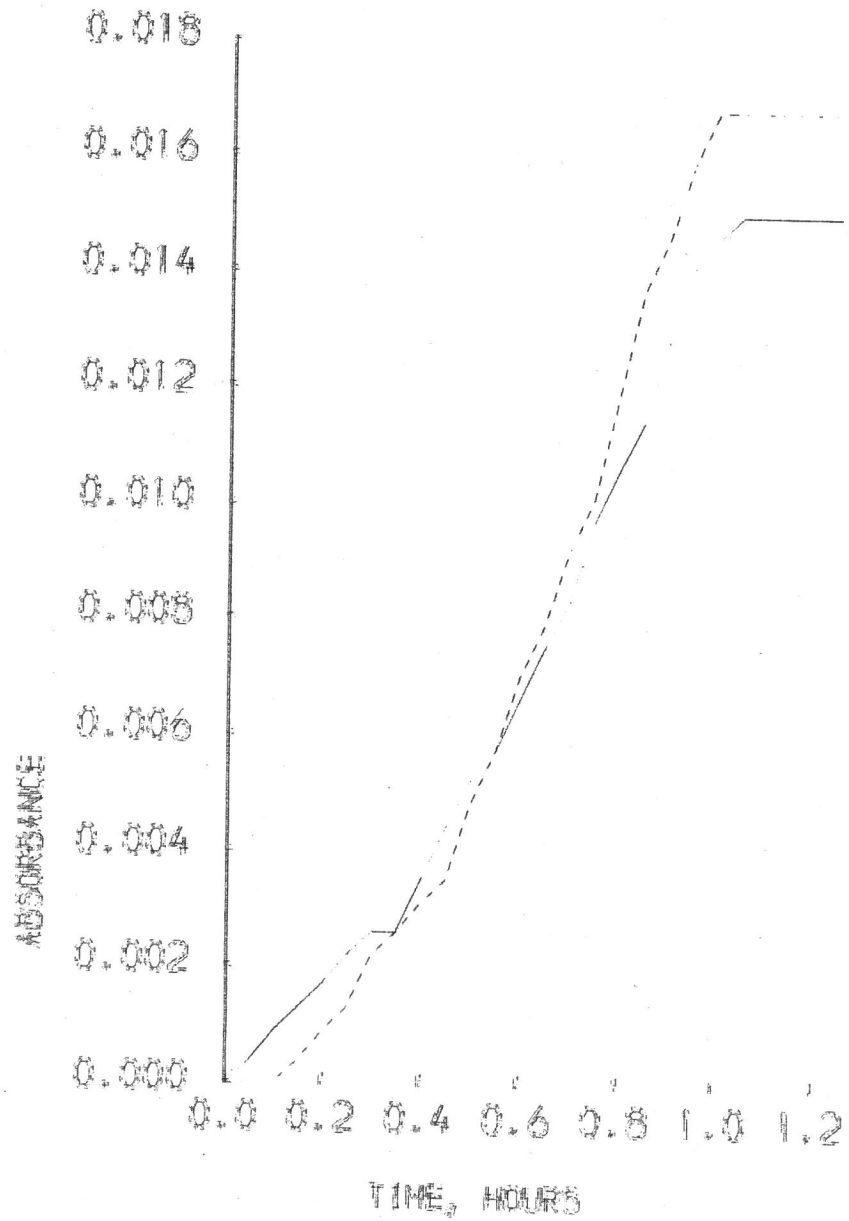


FIGURE 8 GROWTH WITH 20 MG PER L SUCROSE. TWO REPLICATES.

substrate added. The slope of the curve of absorbance on time is proportional to dx/dt where

$$dx/dt = \mu x.$$

However since the amount of growth is small compared to the total number of bacteria present, x is almost constant and the slope is therefore proportional to μ which in turn is constant until the substrate concentration becomes limiting. For this reason it is not necessary to convert the absorbance values to logarithms if we are only interested in the small changes occurring when the substrate becomes limiting.

The abrupt cut-off is interpreted as representing the point where all the added substrate has been utilized and in each case it occurs at an absorbance value approximately proportional to the added amount of substrate. The gradual increase observed after this point is most likely due to the concentration effect caused by evaporation from the culture during the sparging. The loss by evaporation was found to be 23% over 7h. Some increase might also be due to the breakdown of rods into cocci. The results indicate that the change from unrestricted growth to cessation of growth by substrate exhaustion occurs within the limits of the measurable points and so no determination of the exact value of K_s, i.e. when the slope of the graph is half that during unrestricted growth, can be made. However, a maximum value of K_s can be

estimated from the amount of substrate remaining at the last point on the curve representing unrestricted growth. The first point after the cut-off is taken to represent the maximum population value and the absorbance at this point will be proportional to the added substrate. The difference in absorbance values at these two points can then be used to calculate the amount of substrate remaining. This quantity must represent a maximum value for K_s. The values so determined are given in table 7. It is important to note that the lowest value, 0.71 mg l^{-1}, also represents the magnitude of error on the values since this difference is derived from a difference on the recorder chart paper of 0.2mm. It is concluded that the value of K_s for both sucrose and glucose is at least as small as 2 mg l^{-1}.

The curves for sucrose utilization show a change in rate after 0.4 h. This might represent induction by sucrose after continued growth on glucose.

Competition with Escherichia coli

This data is the result of a class experiment investigating the competition between E. coli and Arthrobacter globiformis.

Equal suspensions of both organisms were inoculated into 140ml type chemostats and allowed to grow under batch conditions overnight. The medium flow was then started and the relative numbers of the two organisms

was determined by streaking out a sample of the culture on peptone-yeast extract agar and counting the relative numbers of colonies developing. The measurements were made once or twice daily over two to four days. The medium used was mineral base E plus biotin with glucose as the limiting substrate at 0.4 g l^{-1}.

Table 7. Values of saturation constant estimated from uptake experiments.

Figure	Initial substrate concentration mg l^{-1}	Estimated maximum value for K_s of A. globiformis mg l^{-1}	
		dashed line	continuous line
5	200 glucose	(log plot)	13.25
6	100 glucose	2.21	3.02
7	20 glucose	1.65	0.71
8	20 sucrose	1.07	1.20

It was assumed that no interaction occurred between the two bacteria; cross streaks on solid media showed no antagonism or enhancement of growth between them. A computer program was set up by Mr. J.I. Prosser which predicted the competition based on the difference in μ values. The values of μ_{max} were derived from experiments in batch culture and the value of K_s for E. coli was taken to be 3.50 mg l^{-1} (Koch, 1971). The values of K_s for A. globiformis which gave the best fit

to the experimental data are shown in table 8. The results are in approximate agreement with the uptake experiments in that K_s is in the order of 0.5 to 2.0 mg l^{-1} for glucose.

Table 8. Values of saturation constant for glucose estimated from competition experiments.

Conditions		Predicted value of
temperature	dilution rate	K_s of *A. globiformis*
°C	h^{-1}	mg l^{-1}
25	0.14	1.5
25	0.30	ca.0.5
30	0.29	0.5
30	0.30	ca.0.5
30	0.42	> 2.0 *

* In this experiment *E. coli* was the predominating organism; under the other conditions *A. globiformis* predominated.

CONCLUSION

The results have shown the value of having various models of a biological system based on different *a priori* assumptions, since each model can give different parameter values. The differences between these values can then give an indication of the importance or otherwise of the assumptions. In the three models for maintenance

energy it has not been possible to determine which is correct and so any value quoted will depend on the assumptions governing its calculation. This criticism also applies to the calculations of maintenance energy for other organisms most of which has been based on equation (11). The difference between the assumptions has been shown to be of limited importance since the parameter values only differ by about 15% at the most. However greater variance will occur in populations of lower viabilities.

A comparison of the specific maintenance rates of A. globiformis NCIB 10683 and the organisms given in table 1 (following p. 13) shows that A. globiformis falls into the same group as Saccharomyces cerevisiae, Penicillium chrysogenum, Aspergillus nidulans and the Pseudomonas sp. containing the lowest values of μ_e. A. globiformis thus has a specific maintenance rate typical of organisms found in soil and water environments.

The evidence for a minimum growth rate in A. globiformis is inconclusive; investigations at much lower growth rates will be required to elucidate this problem. However the model put forward should be useful in describing minimum growth rate and the change in viability with dilution rate in other organisms. Its applicability to K. pneumoniae has been demonstrated.

The molar growth yield of A. globiformis has been shown

to fall within the range predicted from energy considerations and the metabolism of an organism which involves complete oxidation of glucose. Since yield reflects the efficiency with which an organism utilizes its substrate it can be concluded that *A. globiformis* is reasonably efficient in using glucose. This is important for an organism found in nutrient-poor environments; it needs to make the best use of its available supply.

The effect of temperature on maintenance energy has been demonstrated. The marked decrease with temperature may be significant for an organism which naturally would experience much lower temperatures than used in most of the laboratory experimentation in this and other studies.

The estimated value of K_s for glucose for *A. globiformis* has been shown to be lower than for other organisms such as *Escherichia*, K_s = 4.0 mg l^{-1}, *Aspergillus*, 5.0 mg l^{-1}, and *Saccharomyces*, 25.0 mg l^{-1} (values from Pirt, 1975). However, Shehata and Marr (1971) have reported a value of 12.6 mg l^{-1} in *Escherichia coli* at high glucose concentrations but a value of 0.068 mg l^{-1} at low glucose concentrations. This might be due to the fact that this particular strain used had been grown for an extended period under glucose limitation which would select for mutants with a low K_s value. The advantage of a low K_s value for a soil microorganism has already been discussed (p. 52).

EFFECT OF NITROGEN LIMITATION

Table 9 shows the results for cell yield and viability determinations in Arthrobacter globiformis NCIB 10683 with ammonium sulphate as the growth-limiting substrate and glucose in excess.

Viability

The viabilities of the nitrogen-limited populations were found to be slightly higher than those under carbon-limitation. A similar situation is found in Klebsiella pneumoniae where carbon (glycerol) - limited populations are less viable than nitrogen (ammonium) - limited populations (Postgate, 1973).

As for carbon-limited cells (see p. 41) equation (14) was fitted to the data by linear regression analysis. The calculated values were :-

r^2 = 0.8411 (significant with $p < 0.05$)

V_{max} = 0.974 (se 0.011)

μ_{min} = 0.00055 (se 0.00017)h^{-1}

The value of V_{max} is higher, and the value of μ_{min} about two times smaller than that under carbon limitation.

Cell yield

The cell yield determinations show a marked increase as the dilution rate is lowered which is contrary to that found under carbon-limitation. This is due to the formation of intracellular polysaccharide though at and below D = 0.027h^{-1}

Table 9. Yield and viability of nitrogen-limited Arthrobacter globiformis

Dilution rate	h^{-1}	0.34	0.27	0.092	0.027	0.0095	0.0074
Viability	%	98.3		97.4	93.6		91.4
(s.d.)		2.5		3.3	2.9		5.6
Cell Yield	mg l^{-1}	136.9	136.6	151.0	207.7	300.2	250.2
(s.d.)		7.7 (6)*	4.1 (2)	4.9 (9)	3.7 (10)	4.3 (7)	13.7 (8)
Unused glucose	mg l^{-1}	505.6	28.8**	393.4	152.9	75.0	50.4
Yield factor	g g^{-1}	0.704	-	0.493	0.380	0.480	0.385

* Figures in brackets refer to the no. of replicates.

** Medium contained 500 mg l^{-1} glucose instead of usual 700 mg l^{-1}.

some of the increase must be accounted for by another constituent, probably cell wall material (see chapter 4).

From the measurements of glucose remaining in the media, it is possible to calculate the glucose used and so give an approximate value for the yield factor in terms of cell mass formed per mass glucose used and these are given in table 9. The value of $0.704 gg^{-1}$ at $D = 0.34h^{-1}$ is greater than the calculated of Y_{max} for carbon-limited cells given in table 5 (see p. 46). This may be due to the difference in composition between carbon-limited and nitrogen-limited cells since the latter contain a high proportion of polysaccharide which will require a lower energy of synthesis than the other cell constituents as it is built up from preformed units, i.e. glucose. If this is so then this will progressively affect the yield factor as the dilution rate decreases and the proportion of polysaccharide increases, and hence any determination of the maintenance energy based on cell yield is made difficult.

That the maintenance requirement is having an effect on the yield is shown by the reduction in yield at dilution rates below $0.34h^{-1}$. Some approximate calculations of the specific maintenance rate based on equation (14) and using various assumed values of Y_{max} suggested that it is from two to six times larger than μ_e for carbon-limited cells but that below $D = 0.027h^{-1}$ it decreases and is not significantly greater than the corresponding μ_e values for carbon-limited cells. The increased specific maintenance rate in nitrogen-

-limited cells may be evidence of "uncoupling" or wastage of the readily available energy supply, but this must be very small at the lower dilution rates.

CHAPTER 3. TECHNIQUES USED IN CHEMICAL ANALYSIS OF CELLS

General

Preparation of cell suspensions. Quantities of cells for analysis were measured out by volume from the chemostat overflow, the bacterial density having been found in the cell yield determinations. For some analyses it was necessary to concentrate the suspensions by centrifugation (25 mins at 4,000 g in a Mistral 2 L centrifuge, Measuring and Scientific Instruments Ltd.) and resuspension in a smaller volume of deionized water. All procedures were performed below 5°C and samples not analysed immediately were stored at -15°C.

The glassware used in analysis was cleaned in a Decon 90 solution (BDH Chemicals Ltd; 30 ml concentrate l^{-1}) containing tincture of Merthiolate (Eli Lilly and Co. Ltd.; 14 ml l^{-1}) to prevent biodegradation of the detergent. The glassware was then rinsed three times in tap water, twice in deionized water and dried at 105°C.

Spectrophotometer techniques. For the colorimetric determinations three subsamples were prepared. After colour development the absorbance was measured in one cm glass curvettes (Thermal Syndicate Ltd.) in a CE 373 linear readout grating Spectrophotometer (Cecil Instruments). A set of four standards were run in each determination plus a water blank.

Carbohydrate

Intracellular polysaccharide and cell wall polysaccharide were measured together as "total carbohydrate" using the anthrone reagent (Herbert, Phipps and Strange, 1971). The reagent was stored at -15°C without any deterioration. Some samples fell outside the region where Beer's law is obeyed (up to 100 μg per sample) so a calibration curve was constructed (see figure 9). The standard used was glucose at 100 μg ml^{-1} in 0.15% benzoic acid.

For cells grown under carbon-limitation at dilution rates less than 0.30 h^{-1} it was not necessary to wash them free of unused substrate since the level of substrate was very low due to the low value of the saturation constant (K_s, glucose, 2.0 mg l^{-1}; see p. 59). In carbon-limited cultures where some glucose remained and in nitrogen-limited cultures, samples were centrifuged in the super-speed unit of the Mistral 2L (15 mins at 20,000g and 0°C) and the centrifugate analysed to give the glucose concentration of the media. Subtracting this from the value of the total suspension gave the carbohydrate content of the cells which accounted for between 10% and 76% of the total depending on the dilution rate.

Polysaccharide

Polysaccharide was isolated from cells by the ultrasonic disintegration of ca. 1 g wet-weight cells suspended in 10ml deionized water in an MSE disintegrator (Measuring and Scientific Equipment Ltd., London) at 20 kc s^{-1} for

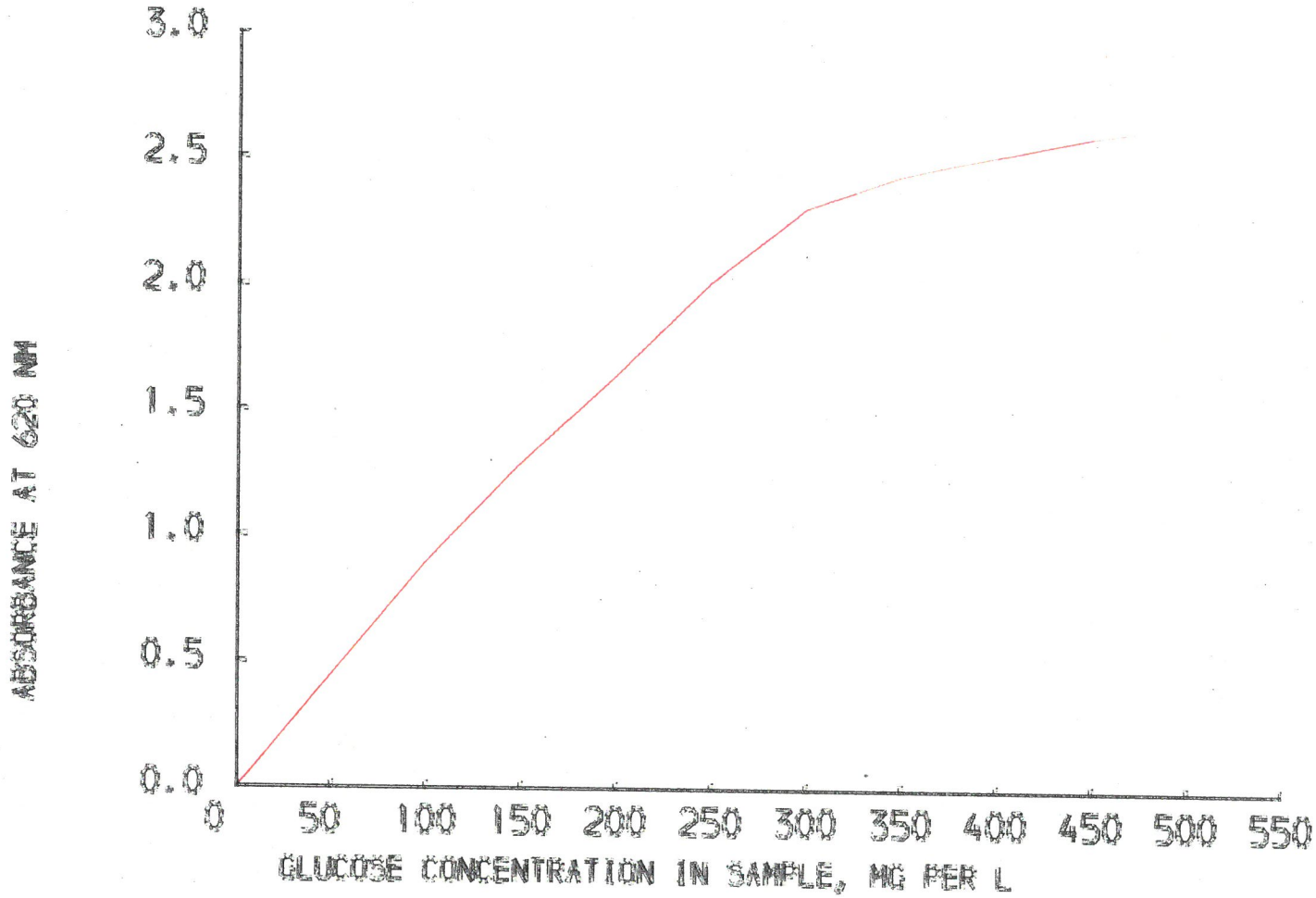


FIGURE 9 CALIBRATION CURVE FOR GLUCOSE IN ANTHRONE REACTION SHOWING DEPARTURE FROM BEER'S LAW AT CONCENTRATIONS OVER 100 MG PER L

2h and at < 10°C. This resulted in almost complete disruption of cells and the cell debris was removed by centrifugation (20,000 g for 30 mins). The protein was precipitated by addition of trichloroacetic acid to 2.5% and then centrifuged (20,000 g for 30 mins). The centrifugate was dialysed for 24h in cellophane against running tap water and then evaporated *in vacuo* to a small volume. The polysaccharide was precipitated by the addition of 2 volumes of ethanol, centrifuged, washed with ethanol, then ether and dried at 100°C (Mulder *et al.*, 1962).

Protein

Protein was determined with the Folin-Ciocalteu reagent (Lowry *et al.*, 1951). A solution of bovine serum albumin, 300 mg l^{-1}, stored at 4°C, was used as the standard. Before analysis the cells were suspended in 1N sodium hydroxide solution and heated in a water bath at 100°C for 15 mins. A mixture of equal volumes of the standard and 2N sodium hydroxide solution was heated at the same time. Cell debris was removed by centrifugation (15 mins at 20,000 g). As the sample was then in alkali, sodium hydroxide was not required in the sodium carbonate solution of the Folin-Ciocalteau reagent (Boylen and Ensign, 1970b). Absorbance was measured at 750 nm.

Deoxyribonucleic acid

DNA was measured using the diphenylamine reaction

(Burton, 1956) using deoxyribose as the standard and following the procedure of Herbert et al. (1971). The time required for complete acid extraction of the DNA (55 mins at 70°C in 0.5 N perchloric acid) was determined by experiment (see figure 10).

It was necessary to convert the deoxyribose values to DNA values. The quantity of DNA can be calculated from its general formula (Herdman, pers.com.) since only purine-deoxyribose is involved, pyrimidine deoxyribonucleosides scarcely reacting. The calculation is slightly affected by the guanine-cytosine ratio so this was assumed to be 63.5%, the value determined by Bousfield (1972) for Arthrobacter globiformis NCIB 8907 which is phenetically very close to A. globiformis NCIB 10683 (see p. 17). The calculated conversion factor was 4.848 g DNA per g deoxyribose.

Ribonucleic Acid

RNA was determined using the orcinol reagent which measures purine-ribose. The major difficulty with this method is interference from hexoses which produce a colour with the orcinol reagent at 670 nm though less so than pentoses. The extent of interference varies with the amount of hexose extracted which can depend critically on the extraction procedure used and so several procedures were tested.

Extraction of RNA. Extraction by hot perchloric acid

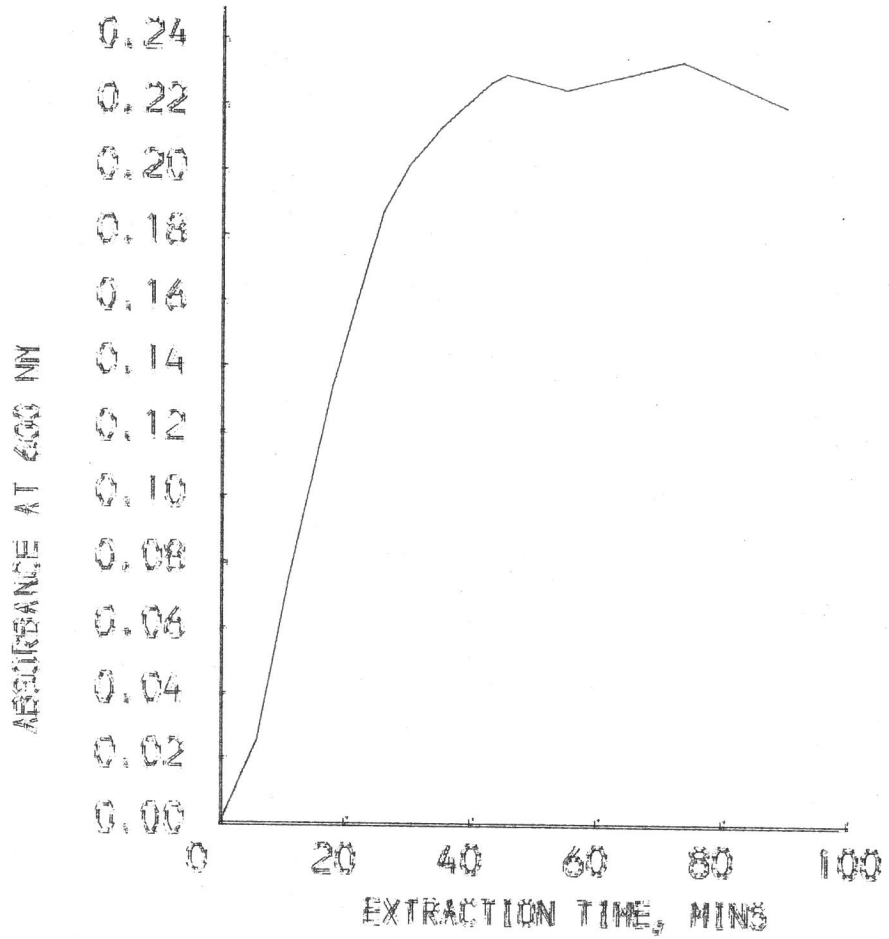


FIGURE 10 EFFECT OF TIME ON THE EXTRACTION OF DNA FROM ARTHROBACTER GLOBIFORMIS BY 0.5N PCA AT 70 C. DNA MEASURED BY DIPHENYLAMINE REACTION.

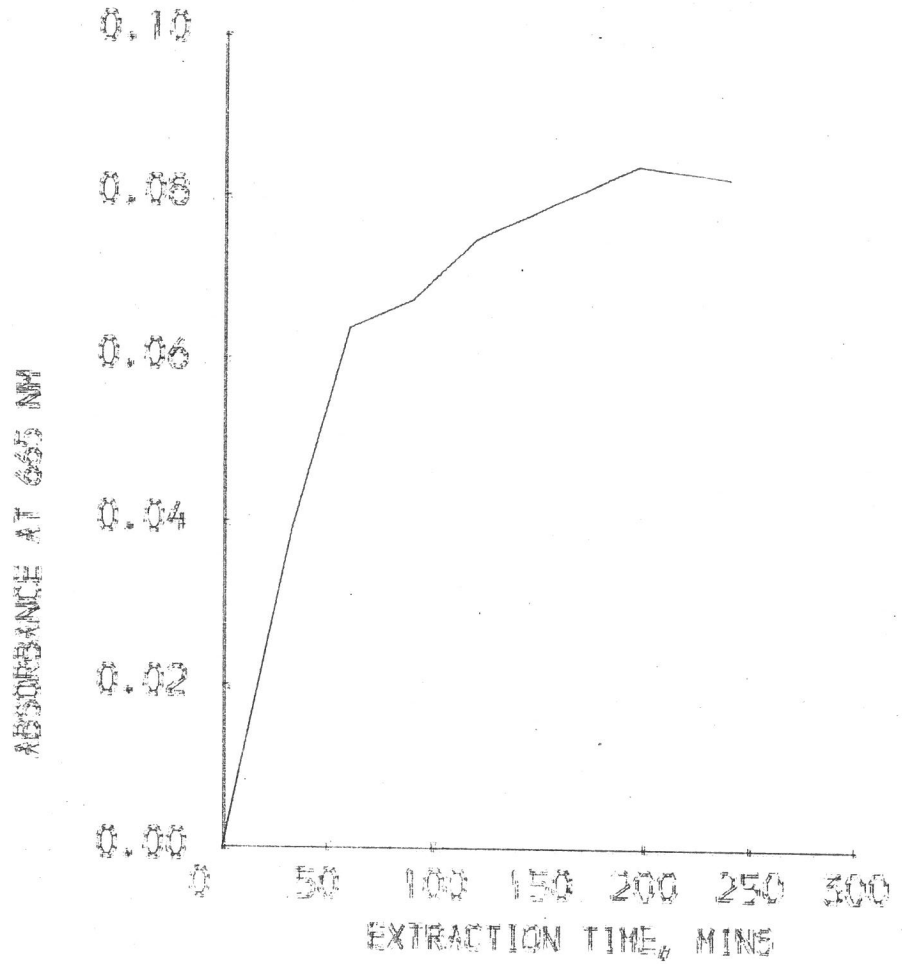


FIGURE 11 EFFECT OF TIME ON THE EXTRACTION OF RNA FROM ARTHRO-BACTER GLOBIFORMIS BY 0.5N PCA AT 37 C. RNA MEASURED BY ORCINOL REACTION.

(0.35 N PCA for 55 mins at 70°C) after Schneider (1945) produced interference in the orcinol reaction which could be seen visually. Dische (1955) suggested applying a correction by taking dichromatic readings at 665 nm and 565 nm but an examination of the complete spectra of treated samples and ribose and glucose standards showed that the assumptions in the correction were false. However the ratio of the absorbances (E665/E565) gives an indication of the degree of interference since hexoses absorb most at the lower wavelength and pentoses at the higher wavelength. This extraction method gave E665/E565 = 1.81 whereas E665/E565 for a ribose standard was 2.89. Pre-extraction with cold 95% ethanol produced no change in E665/E565.

Extraction with cold acid (1N PCA for 18 h at 4°C) after Ogur and Rosen (1950) and following the procedure given by Herbert et al. (1971) but omitting the lipid removal step, again gave considerable hexose interference as judged by scanning the absorbance spectrum on a Unicam SP800 spectrophotometer (E665/E565 was 1.60 - 2.06).

The method adopted was that of Trevelyan and Harrison (1956) which uses 0.5 N PCA at 37°C and which is recommended by Herbert et al. (1971) for cells containing carbohydrate. Pre-extraction with cold acid (0.5N PCA for 30 mins at 0°C) produced no change in E665/E565 and reduced the absorbance by 1.3%. This is attributed to the extraction of pool constituents but as the error

involved is small the procedure was not used routinely. The time required for complete extraction of the RNA (3h) was determined by experiment (see figure 11). E665/E565 came to 1.92 indicating that some interference was still present. It was estimated that this could cause an error of up to 14% in the cited RNA value.

Colour development with orcinol. The orcinol reaction followed the procedure of Herbert et al. (1971). However the colour remained stable and clear when only 1.25 instead of 2.75 volumes of n-butanol were added. The absorbance was measured at 665 nm (Dische, 1955). As recommended by Herbert et al. the heating time was reduced from 20 to 10 mins for cells grown under nitrogen limitation and so rich in carbohydrate. This introduces a slight error due to the difference in the rate of colour development between ribose and RNA as the purine-bound ribose needs first to be hydrolysed before it can react. However this error was considered to be less than that arizing from the doubling in hexose interference occurring after 20 mins heating.

The purine-ribose values determined were converted to RNA values by a conversion factor determined from the general formula of RNA (Herdman, pers.com.) assuming the same guanine-cytoside ratio as for the DNA, a purine-pyrimidine ratio of 1.0 and uracil in place of thymine. The calculated conversion factor was 4.507 g RNA per g ribose.

Lipid

The lipid content was measured gravimetrically by extraction from 0.5-2.0 g freeze-dried cells. Extraction by hot methanol (5 mins at 65°C) followed by chloroform (20 mins at R.T.) (Herbert et al. 1971) appeared to extract more than lipid (see p.77). The adopted procedure was to use chloroform-methanol at R.T. for 3 h (Shaw and Stead, 1971).

Cell Wall

The cell wall content of the cells was determined gravimetrically on walls extracted from 200 mg freeze-dried cells broken-up in a Mickle disintegrator for 2-3 h at $< 10^{\circ}C$ (Work, 1971). The walls were removed by centrifugation at 20,000 g for 30 mins. Their purity was indicated by the absence of an absorbance peak at 256 nm, characteristic of the cytoplasmic fraction (scanned on a Unicam SP800 spectrophotometer).

CHAPTER 4. EFFECT OF DILUTION RATE ON CELL COMPOSITION

INTRODUCTION

Cells grown slowly in a chemostat might be expected to have a composition more closely related to cells found in soil than cells grown rapidly in continuous culture or in batch culture, especially since many studies have shown that cell composition can change markedly with growth rate (Herbert, 1961; Neidhart, 1963).

The experiments here describe the effect of both carbon and nitrogen limitation on the cell composition and growth parameters of Arthrobacter globiformis NCIB 10683 grown at different dilution rates. The results for cell yield and viability have already been discussed (chapter 2).

METHODS

The methodology for the production of cells is given in chapter 2 and the methods of chemical analysis in chapter 3. Total cell counts were made using a Helber counting chamber (Hawksley Ltd., London) and cell sizes were measured on rewetted air-dried smears at a magnification of 2,500 (Luscombe, 1972).

RESULTS

The composition of carbon-limited cells

The results of the various analyses of carbon-limited cells are given in tables 10 and 11. Table 10 gives the composition as a percentage of the total weight of the population.

Table 10. Composition of carbon-limited Arthrobacter globiformis cells on a percentage basis and its change with dilution rate.

Dilution rate	h^{-1}	(0.31)	0.34	0.29	0.084	0.052	0.027	0.015	0.0095
Protein	%	-	30.0	35.5	38.8	39.6	37.6	36.1	40.2
(s.d.)			0.5 (3)*	1.5 (6)	1.6 (11)	0.8 (3)	1.1 (3)	1.0 (3)	0.4 (10)
Total carbohydrate	%	62.8	34.5	23.0	21.3	20.4	21.7	17.2	16.7
(s.d.)		3.7 (3)	0.6 (3)	1.6 (3)	1.2 (7)	1.4 (7)	0.6 (3)	0.9 (3)	0.5 (10)
RNA	%	-	15.5	15.6	15.5	17.3	-	16.1	14.5
(s.d.)			0.7 (3)	0.2 (4)	0.2 (7)	0.5 (4)		1.3 (2)	0.4 (6)
DNA	%	-	4.15	3.06	5.91	4.12	3.86	3.67	3.07
(s.d.)			0.02(3)	0.06(3)	0.05(6)	0.02(3)	0.05(3)	0.10(3)	0.04(6)
Lipid	%	-	9.92**	-	5.66	-	-	-	-
Cell yield	$mg\ l^{-1}$	68.11	122.6	111.3	105.2	101.2	87.55	93.59	91.76
(s.d.)		6.47(8)	3.5 (6)	0.6 (3)	3.6 (16)	3.3 (13)	4.79(8)	3.16(11)	1.27(17)
Cell number†	$X10^{-8}\ ml^{-1}$	2.38	4.32	4.48	6.40	4.55	3.51	3.16	2.99

* Figures in brackets refer to no. of replicates. ** Hot extraction method.

† Data of Luscombe (1972) except at $D = 0.31\ h^{-1}$ (culture on point of washing out).

Table 11. Composition of carbon-limited Arthrobacter globiformis cells on a weight per cell basis and its change with dilution rate.

Dilution rate	h^{-1}	(0.31)	0.34	0.29	0.084	0.052	0.027	0.015	0.0095
Mean cell mass	fg	286	284	248	164	222	249	296	307
Cell morphology		rods	rods	rods	cocci	cocci	cocci	cocci	cocci
Mean cell volume	μm^3	-	0.57	-	0.31	0.29	-	-	0.30
Mean cell specific gravity	$g\ cm^{-3}$	-	0.50	-	0.53	0.77	-	-	1.02
Protein	fg	-	85.1	88.2	63.8	88.1	93.8	106.9	123.4
Total carbohydrate	fg	179.7	97.9	57.1	35.0	45.4	54.1	50.9	51.3
RNA	fg	-	44.0	38.8	25.5	38.5	-	47.7	44.5
DNA	fg	-	11.8	7.6	9.7	9.2	9.6	10.9	9.4
RNA/protein ratio		-	0.52	0.44	0.40	0.44	-	0.45	0.36
DNA/protein ratio		-	0.14	0.09	0.15	0.10	0.10	0.10	0.08

Table 11 gives the composition by weight of a single, average cell. The values at the dilution rate = 0.31h^{-1} were obtained from a culture which contained a large amount of unused glucose (75.8mg l^{-1}) and was probably unstable (see p. 43).

Protein. The percentage protein content shows little change with dilution rate remaining at about 38%. However, rods which form at dilution rate $> 0.25\ h^{-1}$ at 25°C, have an increased carbohydrate content which lowers the percentage protein content. The level of protein is rather less than that found in other bacteria : Klebsiella pneumoniae contains 54 to 62% (Herbert, 1958) and Acinetobacter calcoaceticus contains 53 to 67% (Abbott, Laskin and McCoy, 1974) protein as the dilution rate decreases in carbon-limited culture. However Bekers et al. (1975) found at $D = 0.2\ h^{-1}$, 42 and at $D = 0.1\ h^{-1}$, 47% protein in Brevibacterium 22L and Boylen and Ensign (1970b) using batch-grown cells of Arthrobacter globiformis ATCC 15481 found 41% in rods and 30% in cocci, although the latter were partly nitrogen-limited.

Carbohydrate. The percentage total carbohydrate content shows a marked decrease from 34.5 to 16.7% as the dilution rate is lowered. The carbohydrate consists of two fractions: the polysaccharide constituent of the cell wall and an intracellular polysaccharide (see p. 81). Duxbury (1973) showed the carbohydrate content of the cell wall in glucose equivalents to be 57.4% for rods and 55.4% for cocci. Hence the cell wall polysaccharide content and the intracellular

polysaccharide content can be calculated as given in table 12. The total carbohydrate content at low dilution rates is mainly in the cell wall but at a dilution rate of 0.34 h^{-1} the intracellular polysaccharide increases to 16.0%.

This is a reflection of the glucose-limited conditions. At low dilution rates the external glucose concentration in the chemostat is very low; if we take K_s to be 2mg l^{-1} (see p. 59) then at a dilution rate of 0.0095 h^{-1} the external glucose concentration becomes 0.05mg l^{-1}. This would result in such a low internal glucose concentration that the glycolytic pathway, which leads to the synthesis of protein, lipid and nucleic acids from Krebs cycle intermediates, would never become saturated. At a dilution rate of 0.34 h^{-1} the external glucose concentration becomes 14 mg l^{-1} and so the internal concentration would be correspondingly higher leading to saturation of the glycolytic pathway. The resulting high levels of fructose 1,6-diphosphate and ATP would stimulate the conversion of glucose 1-phosphate to ADP glucose and hence to the polysaccharide (Shen and Preiss, 1966; Dawes and Senior, 1973). In the case where the external glucose was in large excess (0.31 h^{-1}) even more intracellular polysaccharide was accumulated.

Cell Wall. The cell wall contents measured are high compared to other bacteria though stationary phase Streptococcus faecalis has as much as 38% cell wall (Salton, 1964). Zevenhuizen (1966) using an Arthrobacter sp. found 31.8% wall

Table 12. Cell wall and polysaccharide content of carbon-limited cells of Arthrobacter globiformis.

Dilution rate	h^{-1}	0.34	0.084	0.0095
Cell wall	%	32.3	28.2	27.7
s.d. (2 replicates)		0.8	4.1	1.3
Wall polysaccharide	%	18.5	15.6	15.3
Intracellular polysaccharide	%	16.0	5.7	1.4

Table 13. Elemental analysis of carbon-limited cells of Arthrobacter globiformis.

Dilution rate	h^{-1}	0.34	0.084	0.052	0.0095
Carbon	%	41.12	41.67	40.93	42.09
Hydrogen	%	6.20	6.61	6.46	6.61
Residue	%	9.54	10.25	11.70	8.33

of which 60% was polysaccharide in cells grown in nitrogen-deficient medium. Since Duxbury (1973) found the wall thickness to be practically the same in rods and cocci and taking into account the physical shapes, it might be expected that cocci would have a higher wall content than rods. However, this reasoning does not consider any differences in wall density or in the degree of cross-wall formation.

RNA. The percentage of RNA content of the cells shows little change with growth rate, remaining at ca. 16% which is contrary to the situation found in other bacteria where a marked drop in RNA content occurs as the dilution rate decreases. Klebsiella pneumoniae contains 17% at $0.8h^{-1}$ and 7% at $0.1h^{-1}$, Bacillus megaterium contains 22% at $1.4h^{-1}$ and 8% at $0.1h^{-1}$ (Herbert, 1961) and in Acinetobacter calcoaceticus the total nucleic acid content is 12.0% at $0.6h^{-1}$ and 7.5% at $0.1h^{-1}$ (Abbott et al. 1974). Boylen and Ensign (1970b) found the cocci of Arthrobacter globiformis ATCC 15481 grown at ca. $0.05h^{-1}$ to contain 15% RNA and the rods to contain 21%. However, the latter were grown in nutrient rich culture at 30°C and so the growth rate was probably about $0.55h^{-1}$. It is postulated that Arthrobacter globiformis NCIB 10683 would have an RNA content greater than 16% above $0.34\ h^{-1}$ although a temperature higher than 25°C would be required to achieve this. Stevenson (1962) using a strain of Arthrobacter globiformis showed that during batch-growth in a yeast-soil extract medium logarithmic phase cells ($\mu = 0.50\ h^{-1}$, 28°C) had twice the RNA/protein ratio of stationary phase cells. The RNA/protein

ratio of Arthrobacter globiformis NCIB 10683 (table 11) shows some change when cells from the lowest and highest dilution rates are compared butthis might have been more evident if a higher growth rate could have been used.

Since RNA is essential for protein synthesis, it has been suggested that the RNA content of a cell controls the rate of protein synthesis and hence of cell growth. The hypothesis suggested is that ribosomes work with constant efficiency so that the number of ribosomes is proportional to the growth rate and reach practically zero at low growth rates. This has been shown to be incorrect in that a finite amount of RNA is found in slowly-grown cells. Koch (1971) has termed this "extra" RNA, suggesting it is not used to its full efficiency but represents an anticipatory response of the organism to more favourable environmental conditions. The organism will then be able to synthesise protein, and hence grow at the required rate without the delay caused by RNA synthesis. It appears that Arthrobacter globiformis NCIB 10683 contains a substantial amount of "extra" RNA at low growth rates; ca. 14% at $0.0095h^{-1}$.

DNA. The DNA content of Arthrobacter globiformis NCIB 10683 varies between 3.1 and 5.9% increasing with growth rate which is contrary to the findings for other bacteria. In Klebsiella pneumoniae the DNA content varies between 4.4 and 3.0%, in Bacillus megaterium between 3.3 and 2.4% and in Salmonella typhimurium between 1.5 and 1.0% (Herbert, 1961). Boylen and Ensign (1970b) found the DNA content of both rods and cocci

of Arthrobacter globiformis ATCC 15481 to be 2.0%.

If the quantity of DNA per cell is considered, as given in Table 11, the weight is nearly constant at 10fg. This is to be expected if a cell contains one whole genome plus a fraction of replicated DNA, although the amount in an individual cell will depend on its stage in the division cycle. The quantity appears to be reduced in cells at a dilution rate of 0.29 h^{-1} which are short rods and may be due to a reduction in the average quantity of replicated DNA. At 0.34h^{-1}, an increased DNA content is observed. Here the rods are longer and cells counted as single units in the total count may have contained several cross-walls and hence several nuclei . The presence of cross-walls has already been suggested by the high cell wall content at 0.34h^{-1}. The nearly constant value of DNA is also reflected in the DNA/protein ratio. The data of Stevenson (1962) for a strain of Arthrobacter globiformis similarly shows little change in the DNA/protein ratio when comparing stationary phase and logarithmic phase cells grown in batch cultures.

Lipid. The lipid content of cells grown at D = 0.084 h^{-1} is similar to the value of 5.40% for cocci of Arthrobacter globiformis ATCC 15481 obtained by Koshiw, Boylen and Tyson (1972). The value of 9.92% at 0.34h^{-1} is probably an overestimate; the hot extraction method removed a (lipid?) fraction part of which could not be redissolved after drying and weighing.

All the major components of the cell have been analysed and a summation of the percentages, assuming the lipid content to be 5.7% throughout, shows that at $0.34h^{-1}$, 102.7%; at $0.084\ h^{-1}$, 99.8%; and at $0.0095h^{-1}$, 92.6% of the cell weight has been accounted for.

Physical parameters. The mean cell mass is given in table 11 and shows a reduction in mass from rods to cocci at $0.084h^{-1}$. This is to be expected from the change in morphology which is reflected in the change in mean cell volume (table 11). The mean cell specific gravity is the same for both rods and cocci at $0.084h^{-1}$. This is contrary to the findings of Luscombe (1972) whose cell volume measurements indicated that rods have 3.1 times the volume of cocci at ca. $0.1h^{-1}$. However, considerable errors can occur in measurements of cell size. Cell diameter measurements of nitrogen-limited cells grown at $0.025h^{-1}$ and $0.007h^{-1}$ were 1.03s.d.0.09(28) and 1.16 s.d. 0.17 (158) μm with air-drying (figures in brackets show number cells measured), and 1.16s.d. 0.13 (28) and 1.27s.d. 0.36 (28) μm without the air-drying stage, respectively. A student's T-test showed the 10% decrease which occurs during the air-drying and gives a 25% decrease in volume to be significant ($p < 0.05$).

At dilution rates below $0.084h^{-1}$, the cell mass increases which is contrary to the usual situation in bacteria but explains the decrease in the percentage of DNA. The increase in mean cell specific gravity, the values of which should be treated with caution, would suggest that cocci have a

progressively more compact structure as the growth rate is decreased.

Elemental analysis. Table 13 shows the results of a carbon and hydrogen analysis, kindly performed by the micro-analysis laboratory of the department of Organic Chemistry. There appears to be no change in the gross composition which agrees with the general observation found in continuous culture (Herbert, 1975).

The composition of nitrogen-limited cells.

The results of the analysis are given in tables 14 and 15.

Protein. The percentage protein content shows a marked decrease from 29% to 12% as the dilution rate decreases. This can largely be accounted for by the increased carbohydrate content of the cells but it is probable that some of the decrease is also due to nitrogen being diverted into cell wall material. Although the cell wall content of the nitrogen-limited cultures was not measured, the mucopeptide fraction is approximately represented by the remainder when the other percentages are summed assuming there is no other unidentified cell component. At a dilution rate of $0.092h^{-1}$ the remainder is about 3% but increases to 21% at $D = 0.027h^{-1}$.

Carbohydrate. The increase in the percentage total carbohydrate content from 41% to 65% as the dilution rate decreases is the most significant factor in the composition of the cells under

Table 14. Composition of nitrogen-limited Arthrobacter globiformis cells on a percentage basis and its change with dilution rate.

Dilution rate	h^{-1}	0.34	0.27	0.092	0.027	0.0095	0.0074
Protein	%	28.8	23.6	27.8	14.5	11.8	12.0
(s.d.)		0.1 (3)*	0.1 (4)	0.1 (3)	0.1 (3)	0.2 (6)	0.1 (3)
Total carbohydrate	%	41.6	41.1	49.9	52.1	57.6	64.7
(s.d.)		0.7 (3)	0.7 (4)	0.0 (1)	2.5 (3)	0.6 (4)	0.9 (3)
RNA	%	-	-	11.38	5.56	5.36	-
(s.d.)				0.07(3)	0.15(3)	0.06(3)	
DNA	%	-	-	3.93	2.59	2.28	-
(s.d.)				0.07(3)	0.01(3)	0.05(3)	
Lipid	%	-	-	4.64	-	3.43	-
Cell yield	mg l^{-1}	136.9	136.6	151.0	207.7	300.2	250.2
(s.d.)		7.7 (6)	4.1 (2)	4.9 (9)	3.7 (10)	4.3 (7)	13.7 (8)
Cell number $X10^{-8}$	ml^{-1}	4.23	4.30	9.57	6.16	4.27	-
(s.d.)		0.64(4)	0.82(5)	1.73(3)	1.22(5)	0.53(2)	
Net synthetic rate of carbohydrate	mg (100 mg protein)$^{-1}$ h^{-1}	49.2	47.0	16.5	9.7	4.6	4.0

* Figures in brackets refer to no. of replicates.

Table 15. Composition of nitrogen-limited Arthrobacter globiformis cells on a weight per cell basis and its change with dilution rate.

Dilution rate	h^{-1}	0.34	0.27	0.092	0.027	0.0095	0.0074	0.0032*
Mean cell mass	fg	324	318	158	337	703	-	-
Cell morphology		rods	rods	cocci	enlarged cells	enlarged cells	enlarged cells	enlarged cells
Mean cell volume	μm^3	0.71	-	0.31	0.57	0.51	0.82	1.53
Mean cell specific gravity	g cm^{-3}	0.46	-	0.51	0.59	1.38	-	-
Protein	fg	93.3	75.0	43.9	48.9	83.0	-	-
Total carbohydrate	fg	135	131	79	176	405	-	-
RNA	fg	-	-	18.0	18.7	37.7	-	-
DNA	fg	-	-	6.2	8.7	16.0	-	-
RNA/protein ratio		-	-	0.41	0.38	0.45	-	-
DNA/protein ratio		-	-	0.14	0.18	0.19	-	-

* Equilibrium not fully attained.

nitrogen limitation. The increase can be ascribed to the accumulation of intracellular polysaccharide which can be extracted. Carbohydrate accumulation under nitrogen limitation in continuous culture has been observed in Escherichia coli up to 22.5% at D = 0.13h^{-1} (Holme, 1957), Torula utilis up to 34.2% at D = 0.2h^{-1} (Herbert, 1958), Bacillus megaterium up to 6.8% at D = 0.2h^{-1} (Wilkinson and Munro, 1967) and Klebsiella pneumoniae up to 19.5% at D = 0.24h^{-1} (Tempest et al. 1967) . In batch culture of nitrogen-limited Arthrobacter spp., Mulder et al. (1962) observed carbohydrate contents up to 75% depending on the species and Boylen and Ensign (1970b) found 40% carbohydrate in partially nitrogen-limited cocci of Arthrobacter globiformis ATCC 15481.

As in the case of carbon-limited rods growing with a high external glucose concentration and accumulating polysaccharide through saturation of the glycolytic pathway, it may be considered that the same holds true for nitrogen-limited cells where an excess of glucose is always present in the medium although the amount decreases with decreasing dilution rate (Table 9). Holme (1957) found the net synthetic rate of bacterial glycogen in Escherichia coli to increase when the dilution rate decreased but in Arthrobacter globiformis NCIB 10683, the net synthetic rate decreases (see table 14). This indicates that the rate of polysaccharide accumulation does not continue independently of the dilution rate but decreases with it.

Polysaccharide. The intracellular polysaccharide was extracted from nitrogen-limited cells grown at D = 0.092h^{-1} and obtained as a white powder which gave practically no colouration with iodine. The polysaccharide was hydrolysed in 1 N sulphuric acid for 16h at 100°C, the acid removed with barium carbonate and the hydrolysate taken to dryness in a rotary evaporator. The trimethyl silylate derivatives of the hydrolysate were kindly analysed by Dr. J.D. Thornton on a PYE series 105 gas chromatograph. The major constituent (83% of the chart response) was found to be glucose and most of the remainder was rhamnose and galactose which most probably resulted from contaminating cell wall material which contains a polymer of these three sugars (Duxbury, 1973). It is concluded that the intracellular polysaccharide is a polyglucose very similar to those described by Ghosh and Preiss (1965) and Zevenhuizen (1966) for various Arthrobacter spp..

RNA. There is an apparent decrease in the percentage RNA content of the cells due to the increased carbohydrate content. The actual amount per cell increases particularly at D = 0.0095h^{-1} (table 15) but this may well be due to an over-estimate of the ribose by increased interference by glucose (see p. 68). The RNA/protein ratio is similar to that found in carbon-limited cells.

DNA. The percentage level of DNA is much less than in carbon-limited cultures due to carbohydrate accumulation but the

actual amount per cell is similar to that found in carbon-limited cells except at D = 0.0095h^{-1} (table 15). This again is probably due to some cells having cross-walls and so having more than one set of genetic material. Cells having up to four compartments have been observed at this dilution rate (see figure 12g). The DNA/protein ratio is higher than in carbon-limited cells due presumably to the cell not being able to sacrifice DNA whereas the protein is reduced.

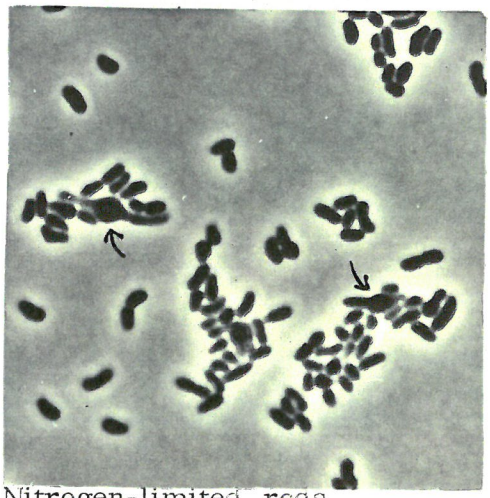
For cells limited by nitrogen, it is to be expected that the amounts of nitrogen found in the protein, RNA, DNA and cell wall would equal that in the input medium, assuming that no nitrogen is lost or fixed. This was true within the limits of experimental error, although the calculations suggested that the 14.5% protein at D = 0.027h^{-1} is low.

Lipid. No increase in the lipid content was noted in nitrogen-limited cells as compared with carbon-limited cells indicating that lipid is not accumulated under glucose excess.

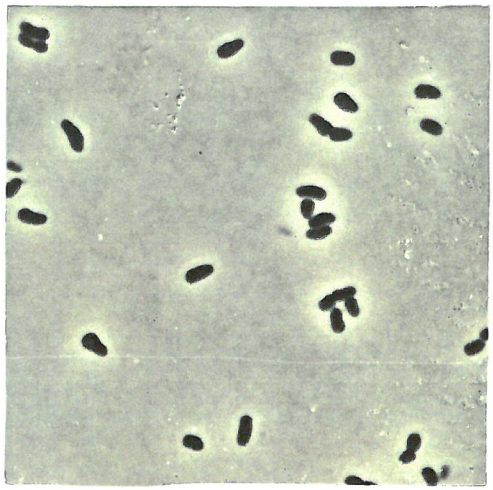
Physical parameters. Nitrogen-limited rods and cocci at D = 0.092h^{-1} have a similar mean cell mass to carbon-limited cells under the respective dilution rates but an increase in mass is noted at D = 0.027h^{-1} and much more at D = 0.0095h^{-1}, attributable to the carbohydrate accumulation.

The mean cell volumes also parallel those under carbon-limitation down to a dilution rate of D = 0.092h^{-1}. They then

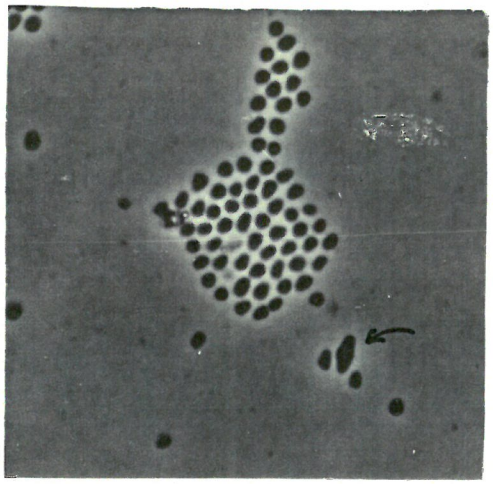
Figure 12. Photographs showing size and morphology of Arthrobacter globiformis under carbon and nitrogen limitation at a range of dilution rates. X2050.



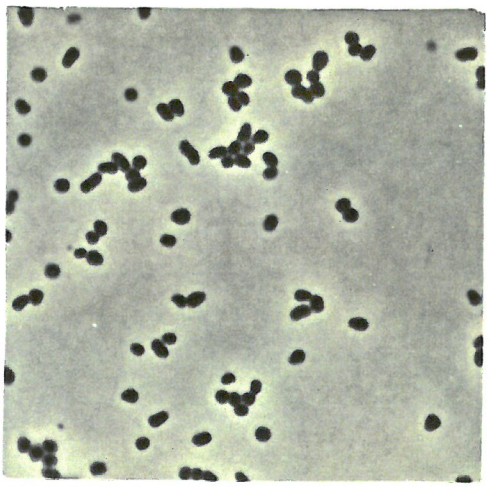
a) Nitrogen-limited rods
$D = 0.35\ h^{-1}$
Arrows show enlarged cells with germinating rod.



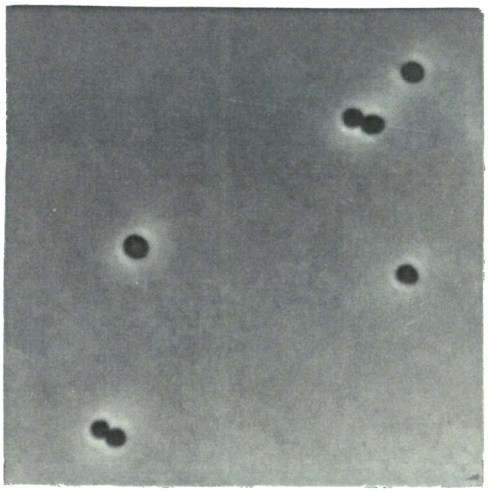
b) Carbon-limited rods
$D = 0.35\ h^{-1}$.



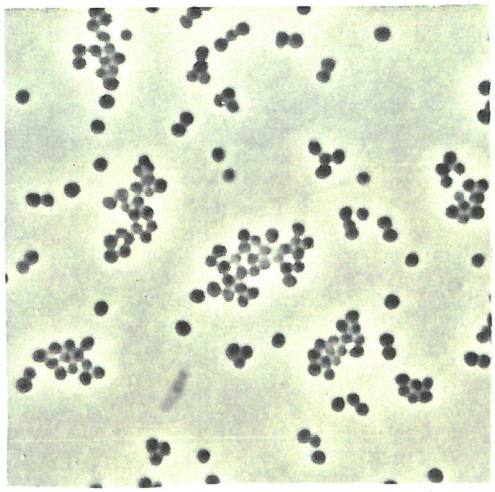
c) Nitrogen-limited cocci/rods.
$D = 0.1\ h^{-1}$.
Arrow shows enlarged cell.



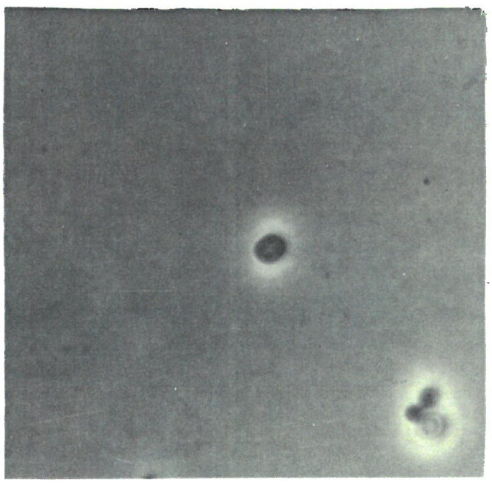
d) Carbon-limited cocci/rods.
$D = 0.1\ h^{-1}$.



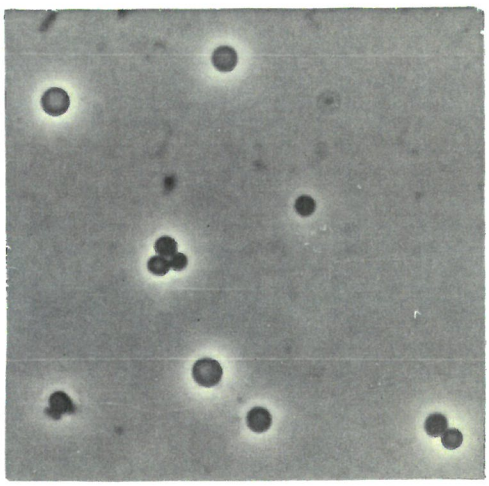
e) Nitrogen-limited enlarged cells.
D == 0.01 h^{-1}.



f) Carbon-limited cells.
D = 0.05 h^{-1}.



g) Nitrogen-limited cells.
D = 0.01 h^{-1}.
Shows cross-walls in enlarged cell.



h) Nitrogen-limited cells, enlarged.
D = 0.003 h^{-1}.

show a progressive increase, as the polysaccharide is presumably packed into the cells, resulting in enlarged cells at D = 0.0032h^{-1}, approximately five times the volume of cocci at D = 0.092h^{-1}. Figure 12 shows cells grown at various dilution rates under both nitrogen and carbon limitation. Figure 13 shows the range of cell diameters of enlarged cells grown at D = 0.007 h^{-1}. The cells have a mean diameter of 1.16 μm but a few have diameters in excess of 1.5 μm. As in carbon-limited cells, the mean cell density values show an increase as the dilution rate is decreased; this again suggests a more compact structure which might be due to increased cell wall material resulting from cross-wall formation.

DISCUSSION

The implications of the cell composition in the natural environment will be discussed in chapter 7.

Formation of enlarged cells

Enlarged cells in Arthrobacter, originally termed "cystites" by Jensen (1934), have long been observed in old cultures in rich media. Much confusion has surrounded their mode of formation on involvement in the so-called "life-cycle" of Arthrobacter species. Stevenson (1963) attributed their formation to some form of stress imposed by a nutritional limitation causing weakening of the cell wall and bulging of the cytoplasmic constituents. It is more probable that their formation is due to the accumulation of intracellular polysaccharide

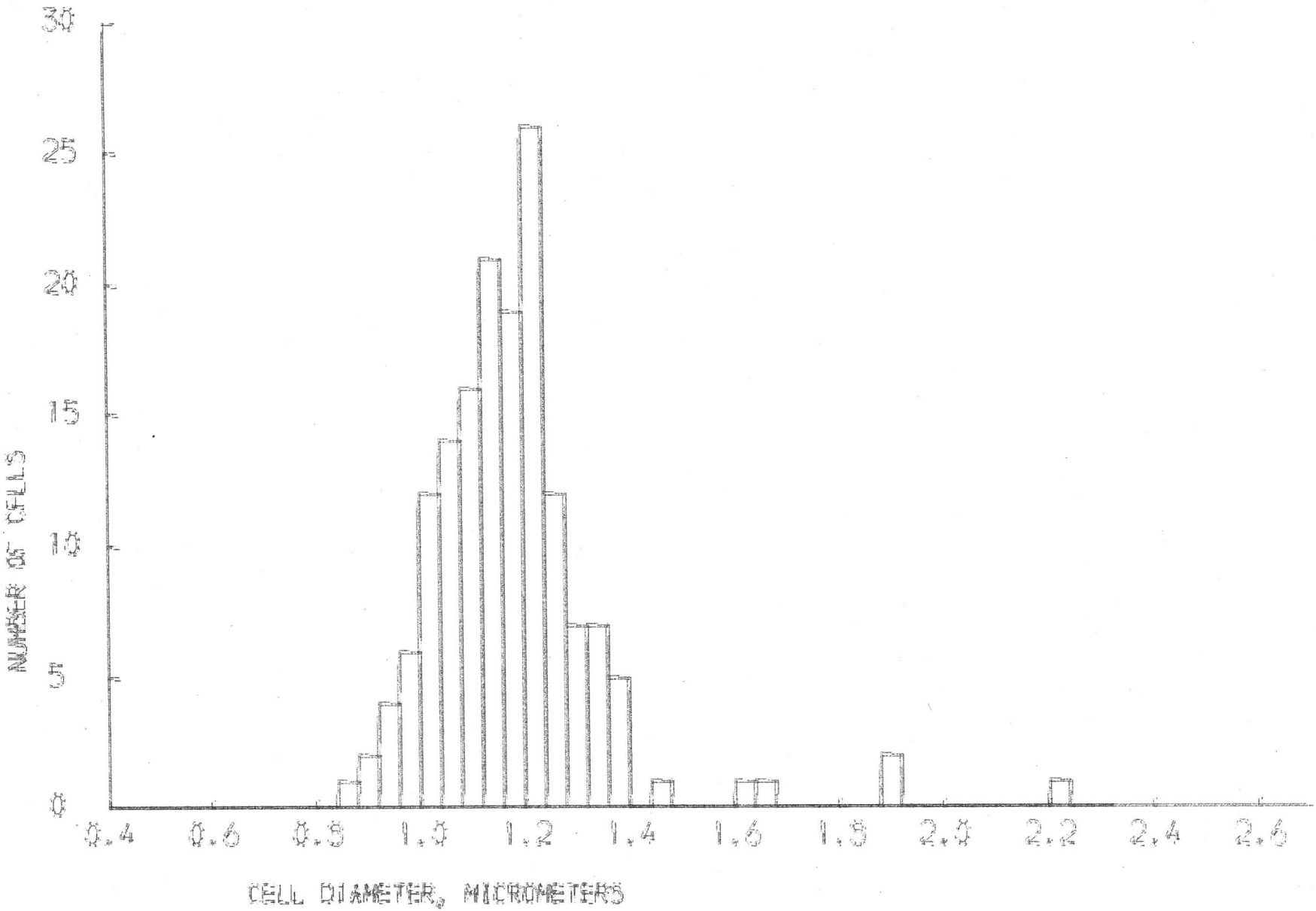


FIGURE 13. HISTOGRAM SHOWING RANGE OF CELL SIZES OF ENLARGED CELLS GROWN AT A DILUTION RATE OF 0.667 PER HOUR

(Mulder and Zevenhuizen, 1967).

The enlarged cells produced in continuous culture under nitrogen-limitation appear to be essentially the same as those produced from old cultures in their size, appearance and the fact that in the presence of a nitrogen source (as in viability determinations), they "germinate" to produce normal rods. One difference is that the population of enlarged cells is over 90% viable and continues to undergo cell division in the enlarged state. This suggests that the cell wall is not weakened in any way. Duxbury (1973) has shown that the thickness, polysaccharide content and resistance to autolysis of the cell wall of batch-produced enlarged cells are the same as those of rods and cocci. Non-viable enlarged cells frequently show one, two or rarely, three protrusions on viability slides without further development - this is interpreted as abortive "germination". The number of "germination tubes" might bear a relationship to the number of cell units formed by the cross-walls (see figure12g) as observed by Stevenson (1963). Enlarged cells with protrusions are also observed at a low frequency at higher growth rates in nitrogen-limited media (see figure 12a and 12c).

It is suggested that enlarged cells are produced in conditions where a carbon source is in excess but where limitation of some other requirement, genetic fault, or inhibition of some metabolic pathway, has slowed down or stopped cell division and allowed this large synthesis of polysaccharide.

CHAPTER 5. EFFECT OF STARVATION ON CELL COMPOSITION

INTRODUCTION

The results in chapter 4 showed that the composition of cells of *Arthrobacter globiformis* depends upon the growth rate of the organism. How the cell composition is affected when the cell is starved of its limiting nutrient is now considered. It would have been useful to examine the changes during starvation in cell populations previously grown at a range of dilution rates but shortage of time necessitated investigating only those populations grown slowly in the chemostat and so more likely to be relevant to the soil environment. Populations of cells grown under both nitrogen and carbon limitation in the chemostat at a dilution rate of $0.01h^{-1}$ (generation time = 69h) were used. In addition cells grown at $0.10h^{-1}$ (generation time = 6.9h) under nitrogen limitation were also investigated.

In these experiments the changes in the population under "minimum stress" (Postgate, 1967) were examined, i.e. when the only major stress was starvation itself. To achieve this it was considered best to hold the organisms in the spent culture medium which has several advantages:

i) Postgate and Hunter (1962) obtained the lowest death rate of *Klebsiella pneumoniae* when it was incubated in its spent culture medium. This was shown to be due to the trace element content.

ii) It eliminates the stresses of centrifugation,

resuspension and temperature shock which can reduce viability (Gossling, 1958).

iii) The population is already at the density (about 5×10^8 ml^{-1}) shown by Harrison (1960) to be optimal for survival in starved K. pneumoniae.

iv) The concentration of limiting substrate is very low in the culture medium and is rapidly reduced to zero, mimicking what is likely to occur in the natural environment.

METHODS

The cultural conditions were as described in chapter 2 and the methods of chemical analysis as in chapter 3. Cell populations were collected from the chemostat overflow, without chilling, and 100ml aliquots were incubated in 250ml flasks at 100 rpm on an orbital shaker (L.H. Engineering Co.) at 25°C. Before each flask was sampled the weight loss by evaporation (not more than 15% after the longest periods) was made up with demineralized water. Flasks were not reincubated after sampling to reduce contamination risks. Three flasks were sampled on each occasion. The chemical analyses were performed on one sample, the cell weight determinations on another and the viability and the oxygen uptake rates on the third.

The population viabilities were measured as described in chapter 2. No correction was applied for the change in total cell numbers due to cell lysis. No visible decrease in cell

density was observed even over the longest incubation periods and Luscombe (1972) showed that the cell lysis rate in A. globiformis NCIB 10683 starved in phosphate buffer at 25°C was very low: a 30% loss in total cell numbers occurred over 70d.

The endogenous metabolism of the starved cells was measured as oxygen uptake, determined by a polarographic method using an electrode of the Clark type (Rank Bros., Cambridge) as described by Luscombe (1972). When the oxygen uptake of starved cultures became too low it was necessary to increase the original cell density to obtain a reasonable electrode reading. The cell density was increased up to ten-fold by rapidly centrifuging the cells, pipetting off a proportion of the centrifugate and resuspending the pellet in the remaining centrifugate. The type of electrode used showed some oxygen uptake with just water in the electrode cell so a correction was applied to account for this which did not exceed 17% of the corrected value. Although weight changes occurred during starvation, the $Q[O_2]$ values are based on the cell density at zero time and also ignore viability changes.

RESULTS AND DISCUSSION

Carbon-limited cells from $0.01h^{-1}$

The results of starvation of carbon-limited cells grown in the chemostat at a dilution rate of $0.01h^{-1}$ are shown in table 16.

Table 16. Starvation of carbon-limited Arthrobacter globiformis cells grown at D = 0.01 h^{-1}. Shows loss of weight of cell constituents compared with zero time (100%).

Time	Cell weight		Total carbohydrate		Protein		RNA		DNA		$Q[O_2]$	Viability	
d	%	(s.d.)	%	(s.d.)	%	(s.d.)	%	(s.d.)	%	(s.d.)	µl O_2 mg^{-1} h^{-1}	%	(s.d.)
0.00	100	1	100	1	100	14	100	3	100	1	22.2	92.7	2.7
0.05	-		-		-		-		-		8.1 0.3	-	
0.12	100		-		-		-		-		5.6	-	
0.9	97.8		-		-		-		-		-	-	
1.2	98.9		99.3	5.7	94.6	2.4	-		102.9	6.3	-	-	
2.4	95.6		-		93.1	3.0	-		95.8	2.6	-	-	
4.2	95.6		89.4	0.7	96.3	8.4	99.3	2.9	99.1	4.1	-	-	
6.8	96.7		84.0	0.4	86.4	2.7	98.8	5.4	93.3	6.4	0.7	-	
11	97.8		86.9	3.9	80.1	1.3	-		99.3	1.8	-	-	
15	89.1		78.0	0.4	76.4	1.6	94.6	2.6	-		-	-	
22	93.5		72.7	2.1	70.9	0.4	87.9	2.1	88.3	2.6	1.1	89.6	5.8
41	83.7		79.4	2.5	70.5	0.9	71.0	1.7	-		-	-	
56	89.1		65.5	1.1	68.2	1.1	56.6	0.9	-		-	73.1	3.8
70	90.1		-		-		-		-		-	-	
196	-		-		-		-		-		-	3.4	0.9

Loss of viability. Little change was observed after 22d and the population was found to be still 73% viable after 56d. Those surviving after 196d are probably the result of cryptic growth. Luscombe (1972) found the viability of the same organism to be 75% after 56d for cells grown at $0.05h^{-1}$ and starved in buffer at a higher cell density. Boylen and Ensign (1970a) found the viability of both rods and cocci of A. globiformis ATCC 15481 to be >65% after 56d at 30°C.

Decrease in rate of endogenous metabolism. The Q $[O_2]$ values decreased rapidly from about 20 to about 1 $\mu l(O_2)$mg cells^{-1} h^{-1} after 7d and remained at this level for at least 22d. Similar values were obtained by Luscombe (1972) using polarography although he obtained lower values by respirometry. The discrepancy between the two methods was about ten-fold at low Q $[O_2]$ values but only two and a half-fold at higher Q $[O_2]$ values.

It is possible to convert Q $[O_2]$ values into maintenance coefficient (q_e^v) values with glucose as the substrate and hence into specific maintenance rate (μ_e) values (see appendix 4). This would give a decrease in μ_e from $0.016h^{-1}$ to $0.0008h^{-1}$. However at zero time the cells are still actively growing so that part of the respiration is providing energy for growth. From the rate of substrate utilization of cells at $0.01h^{-1}$ a Q $[O_2]$ of 9 can be calculated. As the substrate is exhausted, growth processes will shut down and respiration will decrease to the level required for maintenance. For carbon-limited cultures growing at a dilution rate of $0.01h^{-1}$ the value of μ_e is $0.003h^{-1}$ (figure 4) equivalent to a

$Q[O_2]$ value of 4. The $Q[O_2]$ of the starved population reaches this value after about 6h but then decreases further. Suspensions of old cultures of A. globiformis ATCC 8010 (up to 84d old) maintained a $Q[O_2]$ value of 2-3 after 24h (Bain, 1975). If the $Q[O_2]$ values obtained by respirometry by Luscombe (1972) and by Boylen and Ensign (1970a) are considered (0.34 - 0.10) they show the equivalent μ_e value during starvation to be about $0.0001h^{-1}$.

Change in weight of cell constituents. After 56d starvation a 10% loss in gross cell weight was observed, half of which was due to loss of protein and the remainder due equally to total carbohydrate and RNA. Carbohydrate and protein were degraded at equal rates from 100% at zero time to 66 and 68% respectively after 56d. RNA degradation was not observed until after 7d but it then fell to 57% of the original level after 56d. The fact that the viability remained at 73% illustrates the dispensibility of RNA.

A similar experiment was performed but with the cells being starved at a higher density (8.4×10^9 cells ml^{-1}) for 20d. Examination of the cell-free medium for absorbance at 260nm and change in pH over this period gave negative results suggesting that the RNA was completely degraded and that nucleic acid bases or organic acids are not released into the medium. Boylen and Ensign (1970b) found a comparable loss of RNA from cocci of A. globiformis ATCC 15481 after 30d except that degradation took place from zero time and that 260nm-absorbing

material was released into the medium. This difference might be due to the different growth conditions : the cocci were stationary-phase, partially nitrogen-limited and starved at 30°C.

No significant utilization of DNA was observed until 22d. Changes in lipid were not determined but Kostiw et al. (1972) found the loss in total lipid from cocci of A. globiformis ATCC 15481 starved over 14d to parallel the loss in gross cell weight.

Table 17 shows the change in composition of the starved cells. The unknown material is what remains when the measured constituents have been accounted for and is probably the lipid and the muco-peptide fraction of the cell wall. Gaps in the data were filled by interpolation and the values in brackets should be regarded as approximate only. The calculations show no real change in the proportion of nucleic acids, some decrease in carbohydrate and a major decrease in the proportion of protein. The resulting increase in the proportion of the unknown material also represents an increase in the absolute quantity which may indicate an increase in the mucopeptide and hence of cell wall material.

If the 10% decrease in cell weight over the 56d is taken to represent complete oxidation of cell material for endogenous metabolism, then this is equivalent to a mean $Q[O_2]$ of about 0.06. This is much lower than the polarographic measurements given in table 16 and even lower than the respirometry

Table 17. Starvation of Arthrobacter globiformis under various conditions. Shows change in composition as percentages of the total weight at that time. Brackets show values obtained by interpolation.

Time	Total Carbohydrate	Protein	RNA	DNA	Unknown material	Conditions of growth before starvation
d	%	%	%	%	%	
0.0	17	40	14	3.0	26	
6.8	14	35	14	2.9	33	Carbon-limited, D = 0.01 h^{-1}
22	13	30	13	2.8	41	
0.0	58	12	5.4	2.3	23	
8.7	50	9.5	4.9	2.5	34	Nitrogen-limited, D = 0.01 h^{-1}
20	44	(11)	5.1	2.8	(37)	
0.0	50	28	11	4.0	7.8	
6.0	56	(13)	5.6	2.2	(23)	Nitrogen-limited, D = 0.1 h^{-1}
17	45	17	(6.3)	2.7	(29)	

measurements of Luscombe (1972) indicating that the latter method is probably more accurate.

Nitrogen-limited cells from 0.01 h^{-1}

The results of starvation of nitrogen-limited cells grown in the chemostat at a dilution rate of 0.01 h^{-1} (giving enlarged cells) are shown in table 18.

Loss of viability. The viability decreased to 44% over 20d and to 3% over 39d. However, difficulties were experienced in measuring viability since many slide cultures gave no growth or patchy growth, reminiscent of toxicity effects. The recorded viabilities are therefore minimum values and may have been much larger. Nitrogen-limited populations grown at 0.1h^{-1} achieved a composition after 6d starvation practically identical to that of populations grown at 0.01h^{-1} but remained more viable during subsequent starvation (see p. 93). The 4% viability recorded after 107d probably represents cryptic growth.

Decrease in rate of endogenous metabolism. The few measurements made indicated a decrease in $Q[O_2]$ over 2d, similar to that recorded for carbon-limited cells grown at a dilution rate of 0.01h^{-1}.

Change in weight of cell constituents. After 20d starvation a 20% loss in gross cell weight was observed practically all of which was ascribable to loss in total carbohydrate (37%). A 30% loss of protein, most of which occurred in the initial

Table 18. Starvation of nitrogen-limited Arthrobacter globiformis cells grown at D = 0.01 h^{-1}. Shows loss of weight of cell constituents compared with zero time (100%).

Time	Cell weight		Total carbohydrate		Protein		RNA		DNA		$Q[O_2]$		Viability		External Carbohydrate	
d	%	(s.d.)	%	(s.d.)	%	(s.d.)	%	(s.d.)	%	(s.d.)	µl O_2 mg^{-1} h^{-1}	(s.d.)	%	(s.d.)	%	(s.d.)
0.0	100	1.4	100	2	100	2	100	1	100	0	9.9	3.9	91.4	5.6	100	1
0.1	-		-		-		-		-		8.8	2.4	-		-	
0.2	-		-		-		-		-		6.7		-		-	
1.0	106	0	-		92.0	1.9	103	2	96.1	0.1	1.4	0.2	-		100	0
2.1	93.8	0.4	95.5	6.8	69.3	2.8	100	1	91.1	1.5	2.1	1.4	-		101	3
2.7	98.9	4.8	87.8	0.1	87.0	0.6	83.5	3.8	88.9	2.8	-		-		101	0
4.1	-		98.2	4.2	75.6	3.8	92.5	1.9	105.8	0.1	-		-		99.4	3.7
5.3	97.6	0.4	83.8	0.8	69.9	0.9	86.5	0.9	98.1	0.7	-		-		101	2
8.7	88.5	3.2	77.4	1.7	70.9	3.8	79.7	1.3	95.5	1.9	-		-		109	2
10	-		72.9	0.3	67.4	0.9	79.7	1.2	93.4	0.7	-		-		106	1
12	87.5	0.5	71.2	1.0	72.2	1.3	81.0	7.1	99.0	1.2	-		-		108	1
14	66.2	1.4	-		87.3	6.0	85.7	3.6	102	0	-		-		105	3
15	-		61.6	0.8	74.4	3.8	84.0	2.1	93.2	1.1	-		-		113	2
20	80.7	4.1	63.0	3.0	-		75.9	1.1	97.2	0.0	-		44.4	3.5	119	1
30	82.4	1.2	37.6	2.6	79.7	5.1	77.3	2.6	104.4	1.3	-		-		173	3
39	-		-		-		-		-		-		2.8	0.9	-	
51	62.2	1.5	36.0	0.5	100	3	71.9	1.4	103	1	-		-		164	0
66	55.1	0.9	36.8	0.5	100	2	64.8	0.5	115	0	-		-		120	1
107	-		-		-		-		-		-		4.2	0.8	-	

6d, and a 25% loss of RNA, most of which occurred between 2 and 8d, was also observed after 20d. The small contribution of protein and RNA to the loss in cell weight is due to their low concentration in the nitrogen-limited cells at zero-time (table 17). After 66d starvation, a 45% loss in gross cell weight was observed, again mostly due to the 63% loss in total carbohydrate. By this time a 35% loss in RNA had taken place but there was an apparent increase in protein. The reason for this is unknown and the interpretation is difficult as the viability of the population is uncertain. One possible explanation is that of cryptic growth where a fraction of the cells are growing, deriving their nitrogen for protein from the cell wall or nucleic acid nitrogen of lysed cells. The level of carbohydrate in the external medium showed a significant increase after 15d; this is most likely due to the release of intracellular polysaccharide from lysed cells. No major change in the level of DNA was noted over the whole starvation period.

Reference to table 17 shows that the percentage protein and RNA change little over 20d with a slight increase in the percentage of DNA. At 20d the cells contain only 44% carbohydrate instead of 57% whilst an increase in the unknown material is observed, again representing an increase in the absolute quantity which suggests an increase in cell wall material.

The 20% loss in cell weight over 20d is equivalent to a

mean $Q[O_2]$ value of about 0.3.

Test for nitrogen fixation. One possible explanation of the increase in protein after prolonged nitrogen starvation is that of nitrogen fixation which has been reported in various Arthrobacter spp. (Smyk and Ettlinger, 1963; Cacciari and Lippi, 1973). Nitrogen-limited cultures grown at $0.01h^{-1}$ and starved for different periods were tested for nitrogenase activity using the acetylene reduction test (Postgate, 1972), analysing the gases on a Perkin Elmer F11 gas chromatograph. Extensive studies showed neither nitrogen fixation nor ethylene production.

Nitrogen-limited cells from $0.1h^{-1}$

The results of starvation of nitrogen-limited cells grown in the chemostat at a dilution rate of $0.1h^{-1}$ are shown in table 19.

Loss of viability. A practically linear loss of viability was recorded, decreasing to 72% after 17d and to 42% after 37d starvation.

Decrease in rate of endogenous metabolism. Cells taken directly from the chemostat had a very high $Q[O_2]$ value at nearly 200. Based on the rate of substrate utilization, carbon-limited cells growing at $0.1h^{-1}$ would have a calculated $Q[O_2]$ of 75. The much higher value in nitrogen-limited cells is again indicative of "uncoupled" growth in the presence of an excess energy supply (see p. 63). Over the initial 12h the $Q[O_2]$ decreases rapidly and after 4d reaches a constant value of about 6.

Table 19. Starvation of nitrogen-limited Arthrobacter globiformis cells grown at D = 0.1 h^{-1}. Shows loss of weight of cell constituents compared with zero time (100%).

Time	Cell weight		Total carbohydrate		Protein		RNA		DNA		$Q[O_2]$		Viability		External Carbohydrate	
d	%	(s.d.)	%	(s.d.)	%	(s.d.)	%	(s.d.)	%	(s.d.)	µl O_2 mg^{-1} h^{-1}	(s.d.)	%	(s.d.)	%	(s.d.)
0.0	100	3	100	0	100	1	100	2	100	1	173	1	97.4	3.3	100	0.3
0.06	-		-		-		-		-		142	21	-		-	
0.5	-		-		-		-		-		31.6	1.0	-		-	
0.7	185	1	176	3	100	1	103	1	116	0	35.0	2.1	81.3	5.4	50.6	0.4
1.9	208	1	261	8	110	4	112	2	116	1	20.9	1.4	-		6.4	0.1
3.0	211	1	258	3	107	2	112	2	115	1	12.6	0.4	91.9	4.3	6.7	0.4
3.8	215	2	247	2	107	7	107	2	115	1	6.57	0.47	69.5	5.0	6.9	0.1
4.7	208	1	246	1	106	4	110	3	120	0	-		86.5	5.9	6.1	0.1
6.0	217	1	242	3	-		105	2	119	1	5.28	0.78	79.2	6.1	7.0	0.5
8.9	201	4	180	2	-		107	2	-		-		-		-	
12	191	8	158	1	-		107	4	-		-		77.4	5.8	-	
17	169	3	150	1	101	1	-		115	1	5.71	0.60	72.2	5.1	5.0	0.1
28	152	1	119	2	102	2	-		114	0	3.59	0.69	-		5.1	0.1
37	169	2	95.5	0.7	-		-		121	1	3.31	0.85	41.9	2.5	-	
53	-		-		-		-		-		-		26.2	3.6	-	
71	-		-		-		-		-		-		5.9	1.9	-	

Change in weight of cell constituents. After 6d starvation, a 117% increase in cell weight was observed, 60% of which is accounted for by the 142% increase in total carbohydrate and 40% from an apparent increase in the unknown material. The increase in total carbohydrate is presumably due to the accumulation of intracellular polysaccharide over the initial 2d and a concomitant decrease in the glucose in the external medium (see below). During the subsequent starvation there is a loss of carbohydrate such that it attains its original level after 37d. However the cell weight after 37d is still 169% of the original weight, the 69% being due to an increase in the unknown material. Over 37d there was no change in protein, some increase in RNA and a 15-20% increase in DNA. A 20% increase in DNA during starvation was noted by Boylen and Ensign (1970b) in both rods and cocci of A. globiformis ATCC 15481.

Table 17 shows how the composition is affected by the uptake of glucose and subsequent utilization of the intracellular carbohydrate. After 6d the cells consisted of 56% carbohydrate instead of 50% causing the drop in the percentage of the other constituents except in the unknown material. If this represents an increase in the mucopeptide of the cell wall then one unexplained discrepancy is the source of nitrogen since there is no degradation of the other nitrogen-containing constituents and it would be expected that all the nitrogen in the medium had been exhausted. An alternative explanation is an increase in lipid material but this was not examined. It is noteworthy that these cells, grown at $0.1 h^{-1}$

had, after 6d, the same composition as nitrogen-limited cells grown at $0.01h^{-1}$. After 17d the carbohydrate content had been reduced to 45% causing the rise in the percentage of the other constituents.

Uptake of glucose. The uptake of glucose from the external medium and accumulation as intracellular carbohydrate, presumably as the polysaccharide, was investigated in more detail over the first 46h. The results are shown in figure 14 which gives the decrease in extracellular glucose, the decrease in the total culture carbohydrate (medium plus cells) and the difference between these which is the intracellular carbohydrate. Over the first 10h there is a practically linear change in the values. The net synthetic rate of carbohydrate of 19.2mg (100mg protein)$^{-1}$ h^{-1} is in good agreement with the value of 16.5 calculated for the growing population at a dilution rate of $0.1h^{-1}$ (see table 14). This indicates that even though the cells have been starved of nitrogen the synthesis of polysaccharide continues at the same rate for at least 10h. The rate then decreases, reaching zero somewhere around 46h.

The linear decrease in the total carbohydrate gives an indication of the rate of endogenous metabolism since over this period there is little change in any other constituent; the equivalent $Q[O_2]$ was calculated to be 46. After 10h the rate of decrease of total carbohydrate slows down and over 27-46h the mean $Q[O_2]$ was calculated to be 14. These values agree approximately with those given in table 19 obtained by

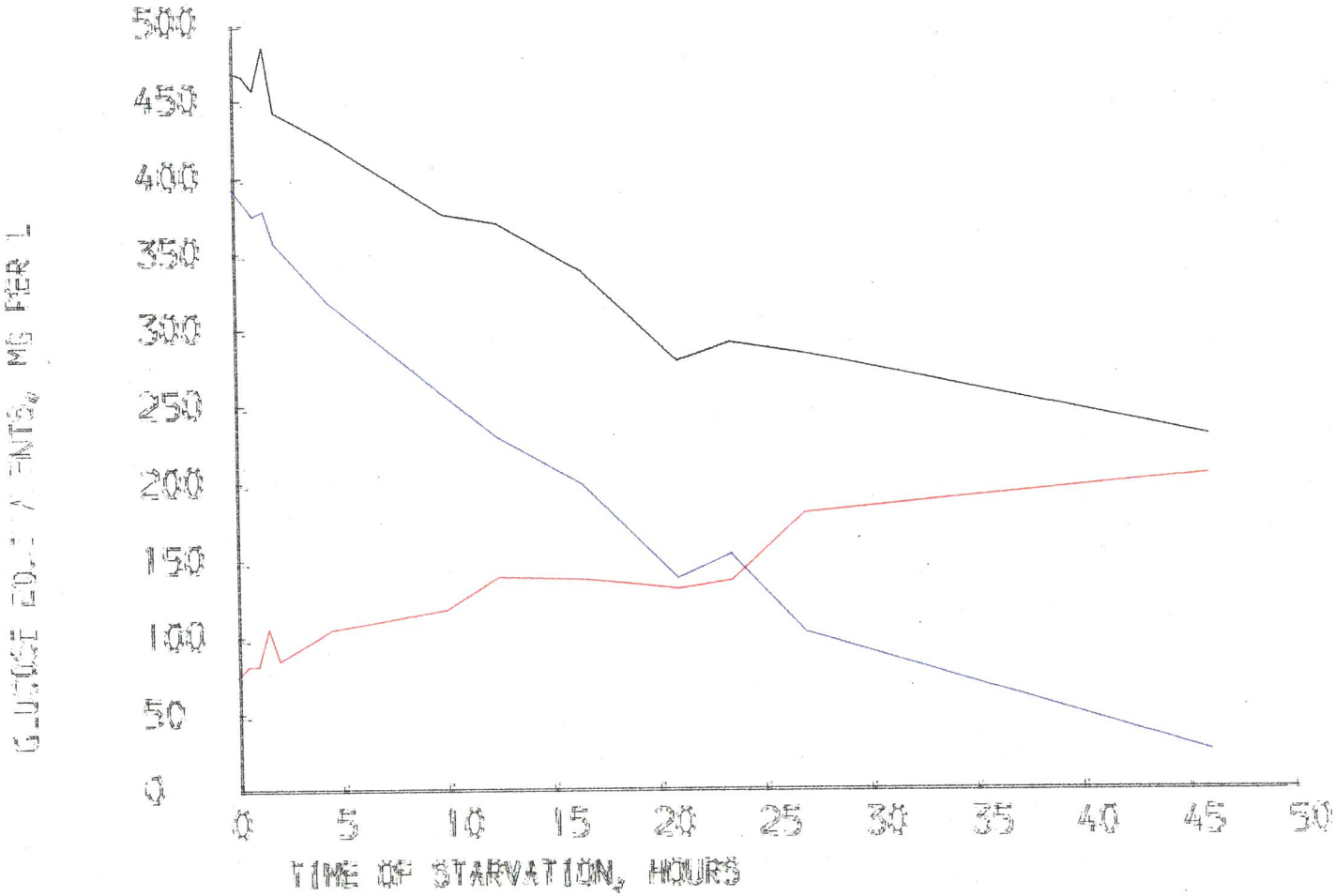


FIGURE 14. UPTAKE OF GLUCOSE BY NITROGEN-STARVED ARTHROBACTER GLOBIFORMIS CELLS (151 MG PER L AT 0 H) GROWN AT 0.1 PER H. RED, TOTAL CARBOHYDRATE IN CELLS. BLUE, EXTRACELLULAR GLUCOSE. BLACK, TOTAL CARBOHYDRATE IN CULTURE (SUM OF RED PLUS BLUE).

polarography. From the decrease in weight from 6-17d given in table 19 the equivalent $Q[O_2]$ was calculated to be about 2 which is slightly lower than the observed values given in table 19.

COMPARISON OF CARBON AND NITROGEN STARVATION

The differences in the behaviour of carbon-limited and nitrogen-limited cells to starvation will now be considered. It is perhaps important to emphasise that the carbon-limited cells have been starved of their carbon (energy) source whereas the nitrogen-limited cells have been starved of their nitrogen source whilst having an exogenous energy supply. In both nitrogen-starved populations carbohydrate was always detectable in the medium throughout the starvation period. This is analagous to the situation that could occur in nature, i.e. the exhaustion of the limiting nutrient but an excess of other nutrients.

The patterns of intracellular substrate utilisation are markedly different in carbon and nitrogen starved cells. Protein is the major substrate in the carbon starved cells with carbohydrate and RNA contributing as well whereas in the nitrogen starved cells carbohydrate in the form of intracellular polysaccharide is practically the sole substrate with only slight contributions from RNA and protein. It is interesting that in the nitrogen starved cells the intracellular polysaccharide appears to be used as a substrate for endogenous metabolism in preference to the glucose in the medium which

remained at 25 mgl^{-1} with the cells from $0.1h^{-1}$ after the initial uptake into polysaccharide and at 75mgl^{-1} with the cells from $0.01h^{-1}$. The actual reason for this is not known but it is possible that the spontaneous breakdown of the polysaccharide provides sufficiently high levels of glucose-1-phosphate and glucose-6-phosphate for use in endogenous metabolism and to inhibit further glucose transport. The protective effect of polysaccharide on the degradation of protein and RNA has also been observed in polysaccharide rich cells of A. globiformis ATCC 15481 (Boylen and Ensign, 1970b; Louie and Klein, 1974) and in many other micro-organisms (see chapter 1).

The loss of protein from both carbon and, to a lesser extent, nitrogen starved cells from $0.01h^{-1}$ may reflect the loss of unrequired protein. Meganathan and Ensign (1972) showed the Krebs cycle enzymes of A. globiformis ATCC 15481 to be stable over 7d whilst catalase and NADH oxidase decreased. Induced enzymes can be lost as shown by the loss of ability to use 2-hydroxypyridine after 3d (Boylen and Ensign, 1970b) although β-galactosidase activity was constant in A. atrocyaneus over 7d (Scherer and Boylen, 1975). The loss of protein from a starving cell may be helpful in that less maintenance energy is required for protein turnover which can proceed at $3\text{-}5\%h^{-1}$ in Escherichia coli (Mandelstam, 1960).

In both nitrogen and carbon starved cells there was a delay of 2-7d before any appreciable RNA degradation took place. This could be advantageous to the organism in that

while it retains its RNA it is able to rapidly start protein synthesis on the reestablishment of favourable conditions just as "extra" RNA is beneficial to slowly-growing cells.

Starved cells from all three conditions showed what is thought to represent an increase in mucopeptide and therefore in cell wall material. This could either be in the form of cross-walls or as additions to the existing wall. Boylen and Pate (1973) in their study of starved A. globiformis ATCC 15481 over 56d showed no evidence of morphological changes from electron microscope photographs but Labeda and Casida (1975) have recorded a slight thickening of the wall of a strain of A. globiformis starved in soil.

The direct measurements of $Q[O_2]$ by the polarographic method, when compared to calculated values based on decrease of cell weight or of carbohydrate and to values measured by respirometry by Luscombe (1972) appear to overestimate the $Q[O_2]$ considerably at low values but give better agreement at values above 10. Luscombe suggested that the error might be due to adhesion of cells to the electrode membrane. Nevertheless, the $Q[O_2]$ measurements all showed an initial rapid decrease to a steady level after 1-3 days. Table 20 summarises the values of $Q[O_2]$ obtained at this steady level by the different methods.

Based on the decrease in cell weight, nitrogen starved cells have five times the $Q[O_2]$ of carbon starved cells from $0.01h^{-1}$ and nitrogen starved cells have an even higher $Q[O_2]$.

Table 20. $Q[O_2]$ and the corresponding maintenance energy values in nitrogen-starved and carbon-starved Arthrobacter globiformis.

Method		Carbon-starved ($0.01\ h^{-1}$)	Nitrogen-starved ($0.01\ h^{-1}$)	Nitrogen-starved ($0.1\ h^{-1}$)
Polarography	$Q[O_2]$	1	1	6
	$\mu_e\ h^{-1}$	0.0008	0.0008	0.005
Respirometry	$Q[O_2]$	0.34		
(Luscombe, 1972)	$\mu_e\ h^{-1}$	0.00026		
Decrease in	$Q[O_2]$	0.06	0.3	2
cell weight	$\mu_e\ h^{-1}$	0.00005	0.00024	0.0016

This is presumably due to the greater availability of an energy supply. Since there is no a priori reason for believing that nitrogen starved cells require a higher input of energy for maintenance and since they do not maintain their viability any better than carbon starved cells then it must be concluded that the endogenous metabolism in the nitrogen starved cells is partly uncoupled.

The $Q[O_2]$ of 0.06 in carbon starved cells is very low; values in starving populations of other bacteria are usually greater than unity (see table 1). Measurements of carbon dioxide evolution in A. globiformis ATCC 15481 gave an equivalent $Q[O_2]$ value of 0.22 (Boylen and Ensign, 1970a) and of 0.0037 in dessicated cocci (Boylen, 1973).

Although difficulties were encountered in the measurement of viability in nitrogen starved cells, the results nevertheless indicate that these cells loose their viability at a much greater rate than carbon starved cells (2.2% d^{-1} compared with 0.4% d^{-1} for cells grown at a dilution rate of 0.01h^{-1}). This could be due to the higher rate of endogenous metabolism in spite of the fact that the nitrogen starved cells had a plentiful energy supply. Cells containing large quantities of endogenous reserve material generally maintain their viability longer than those lacking it (see p. 10 and also Strange, 1968) though exceptions occur as in Micrococcus luteus (table 1). Luscombe (1972) followed the loss in viability of nitrogen and carbon-limited cells of A. globiformis NCIB 10683 under

stress conditions and found that nitrogen-limited cells had better survival ability at pH 4.1 and in sodium chloride solution (a_w 0.90) but worse survival ability in sucrose solution (a_w 0.90) and at 48°C.

An alternative explanation of the increased loss of viability in the nitrogen starved cells is that these cystites have weakened cell walls caused by the accumulation of polysaccharide as has been suggested by Stevenson (1963). However, this is incompatible with the observation of higher viabilities than carbon-limited cultures in the chemostat (table 9 and figure 1).

The implications of these findings to the soil environment will be deferred to chapter 7.

CHAPTER 6. INVESTIGATIONS ON PIGMENTATION

This chapter is a report of what is known of the blue and yellow pigments of Arthrobacter globiformis NCIB 10683.

BLUE PIGMENT

As reported by Luscombe (1972), Arthrobacter globiformis NCIB 10683 produces both an unstable soluble and stable unsoluble blue pigment, particularly at high growth rates. The blue coloration is easily seen during logarithmic batch growth and in the chemostat at dilution rates over $0.2h^{-1}$.

When Arthrobacter globiformis was grown in the chemostat with Escherichia coli in the experiments described in chapter 2 (see p. 57) a considerable increase in blue pigment was observed and heavy deposition on the vessel wall, again more at high dilution rates. This also happens when it is grown with Bacillus subtilis (Gray, pers.comm.) and is similar to the observations of Heumann, Young and Gottlieb (1968) who found increased pigmentation from an Arthrobacter sp. in cross-streaks on solid media with several oxidative micro-organisms including Escherichia coli. They concluded that the Arthrobacter sp. produces the soluble, colourless leuco-indigoidine which is then oxidised to the insoluble blue indigoidine. This is presumably what happens in the continuous culture experiments.

The insoluble pigment could be dissolved in pyridine when it gave an absorption maximum at 606nm. It also dissolved in saturated sodium bicarbonate solution containing sodium dithionite (2%) to give a colourless solution. Rapid aeration

of this gave a transitory reappearance of the blue pigment. It may be concluded that the blue insoluble pigment of Arthrobacter globiformis is in the class of compounds described as indigoidine (Kuhn, Starr, Kuhn, Bauer and Knackmuss, 1965). It is probable that the blue soluble pigment is closely related (Knackmuss and Beckmann, 1973).

YELLOW PIGMENT

Characterisation of pigment

Most of the yellow pigment of Arthrobacter globiformis NCIB 10683 could be extracted, along with lipid, with a chloroform/methanol mixture (2:1 v/v) at room temperature. The absorption spectrum in this solvent showed three peaks at 422, 446 and 476 nm while in 95% ethanol the peaks were shifted to 416, 439 and 469 nm, showing the pigment to be a carotenoid. Thin-layer chromatography on silica gel using petroleum ether, 50:50 petroleum ether/diethyl ether and then diethyl ether failed to move the pigment showing it to be unusually polar. It could be moved using 95% ethanol. It is thus possibly similar to the decaprenoxanthin mono-glucoside and diglucoside isolated from an Arthrobacter sp. by Arpin, Lidden-Jensen and Trouilloud (1972). During cell wall isolation (chapter 3) the pigment appeared to be associated with the membrane fraction.

Arthrobacter globiformis NCIB 10683 readily forms a white mutant, identical in colony morphology apart from the coloration.

No pigment could be extracted from the white cells.

Using cell populations consisting mainly of the yellow wild type and assuming a specific extinction (1%, 1cm) of 2500 (Davis, 1965) at 446 nm, the quantity of pigment in the cells was calculated to be 0.013% (dilution rate = $0.1h^{-1}$) and 0.008% ($0.35h^{-1}$).

Role of the pigment

The function of carotenoid pigments in non-photosynthetic micro-organisms is uncertain although several studies have shown them to confer photoprotection (Kunisawa and Stanier, 1958; Mathews and Sistron, 1959). Tentative evidence has been put forward for their part in protein and membrane stabilisation and also metabolic functions (Krinsky, 1971). Lee and Morrison (1971) have suggested that the light-sensitive carotenoids of Arthrobacter citreus may have a role in electron transport.

Experimental studies. Two simple experiments were set up to determine whether the yellow carotenoid pigment conferred photoprotection by comparing the wild type with the white mutant.

Nutrient agar plates were streaked with a mixed yellow and white population of Arthrobacter globiformis NCIB 10683. Toluidine blue, as a photosensitizer, was added in three levels : 0, 10, 200 µg per plate. The plates were incubated at room temperature over a fluorescent light for 5 days with

half the plates wrapped in aluminium foil. Those incubated in the dark showed full growth except with 200 µg toluidine blue where the growth was just visible. Those incubated in the light showed reduced growth at 0µg, less at 10µg and none at 200µg. All the plates were then incubated 1-3 days in the dark and the numbers of white and yellow colonies counted. The results are shown in table 21. Chi-squared tests applied to the counts showed a significant ($p < 0.005$) increase in the proportion of yellow colonies at 10 µg toluidine blue in the light compared to 0µg in the light and a significant ($p < 0.001$) increase in the proportion of yellow colonies at 10µg in the light compared to 10µg in the dark.

Table 21. Colony counts of white and yellow Arthrobacter globiformis after incubation on nutrient agar.

Level of Toluidine blue µg	Fluorescent Light			Dark		
	white	yellow	%yellow	white	yellow	%yellow
0	47	13	21.7	64	11	14.7
10	168	129	43.5	68	11	13.9
200		no growth		51	8	13.6

In the second experiment inoculated test tubes containing mineral base E ($0.2 g l^{-1}$, glucose) were incubated at room temperature for 2 days and exposed to a total of about 8h direct sunlight. Some tubes contained toluidine blue at $0.5 mg\ l^{-1}$ and half of the tubes were covered in foil. During this time

little growth was observed in the tubes. The suspensions were then streaked onto nutrient agar, incubated in the dark and the white and yellow colonies counted as shown in table 22. Chi-squared tests showed the increased proportion of yellow colonies in the light to be significantly ($p < 0.005$) greater than that in the dark with no toluidine blue. Addition of the photosensitiser appeared to cause complete death in the light.

Table 22. Colony counts of white and yellow *Arthrobacter globiformis* after incubation in mineral medium.

Level of Toluidine blue mg l^{-1}	Sunlight			Dark		
	white	yellow	%yellow	white	yellow	%yellow
0	26	24	48.0	64	34	34.7
	46	42	47.7	117	51	30.4
				21	12	36.4
0.5	no living cells detected			100	52	34.2
				30	21	41.2

Conclusion. Given that the increased proportion of yellow colonies is due to the death of the white mutants, it is concluded that the yellow pigment prevents death by photodynamic action. This action is also increased in the white mutants by the presence of the photosensitizer toluidine blue, although this does in itself have a toxic effect at high concentrations.

Occurrence of the white mutant.

Prolonged studies with Arthrobacter globiformis NCIB 10683 have shown a high frequency of occurrence of the white mutant. Repeated streaking out of manifestly yellow colonies on peptone-yeast extract agar always gave approximately the same proportion of yellow and white colonies; the average percentage of yellow being 93.6. Taking p to be the probability of mutation at one division, y to be the proportion of yellow wild type cells in a colony and n to be the number of generations, it can be easily shown that :

$$y = (2 - p)^n / 2^n$$

Using this, p was calculated to be 0.0040 mutations per division. (This assumes that the viability and growth rate of the two types are equal). This rate is much larger than observed rates for spontaneous mutation, 10^{-5} to 10^{-7} mutation per division, suggesting that the mutation is not spontaneous but possibly due to the loss of genetic material in the form of an episome or phage. A bacteriophage has been found associated with a similar pigmented organism, A. polychromogenes (species incertae sedis), by Schippers-Lammentse, Muijsers and Klatser-Oedekerk (1963). The back mutation has never been observed.

During cultivation in the chemostat under the various conditions used in this study the ratio of white to yellow cells was monitored. Under carbon limitation at dilution

rates below $0.1h^{-1}$ a significant decrease in the yellow population was observed whereas above $0.1h^{-1}$ the ratio was constant over at least 19 generations. Although these observations were made on cultures exposed to diffuse light, light does not appear to be the cause as a culture grown at $0.05h^{-1}$ in the dark showed no significant change, the reverse of that expected. Under nitrogen limitation, the ratio was generally constant except at dilution rates below $0.007h^{-1}$, although the pattern was not as consistent as under carbon-limitation. Figure 15 shows the changes in ratio under various conditions.

During prolonged starvation of populations from nitrogen and carbon limited cultures grown at $0.01h^{-1}$ the ratio was constant showing no differences in starvation resistance and Luscombe (pers.comm.) reported no differences in resistance to various stress conditions (low pH, low water availability, high temperature). In this study, no differences between the cell yield or viability measurements in yellow and white populations were observed.

The reason for the selection for white at low growth rates is thus unknown and requires further investigation. It is possible that the white mutant has a slightly lower Ks value for the limiting substrate giving it a selective advantage at the lower growth rates.

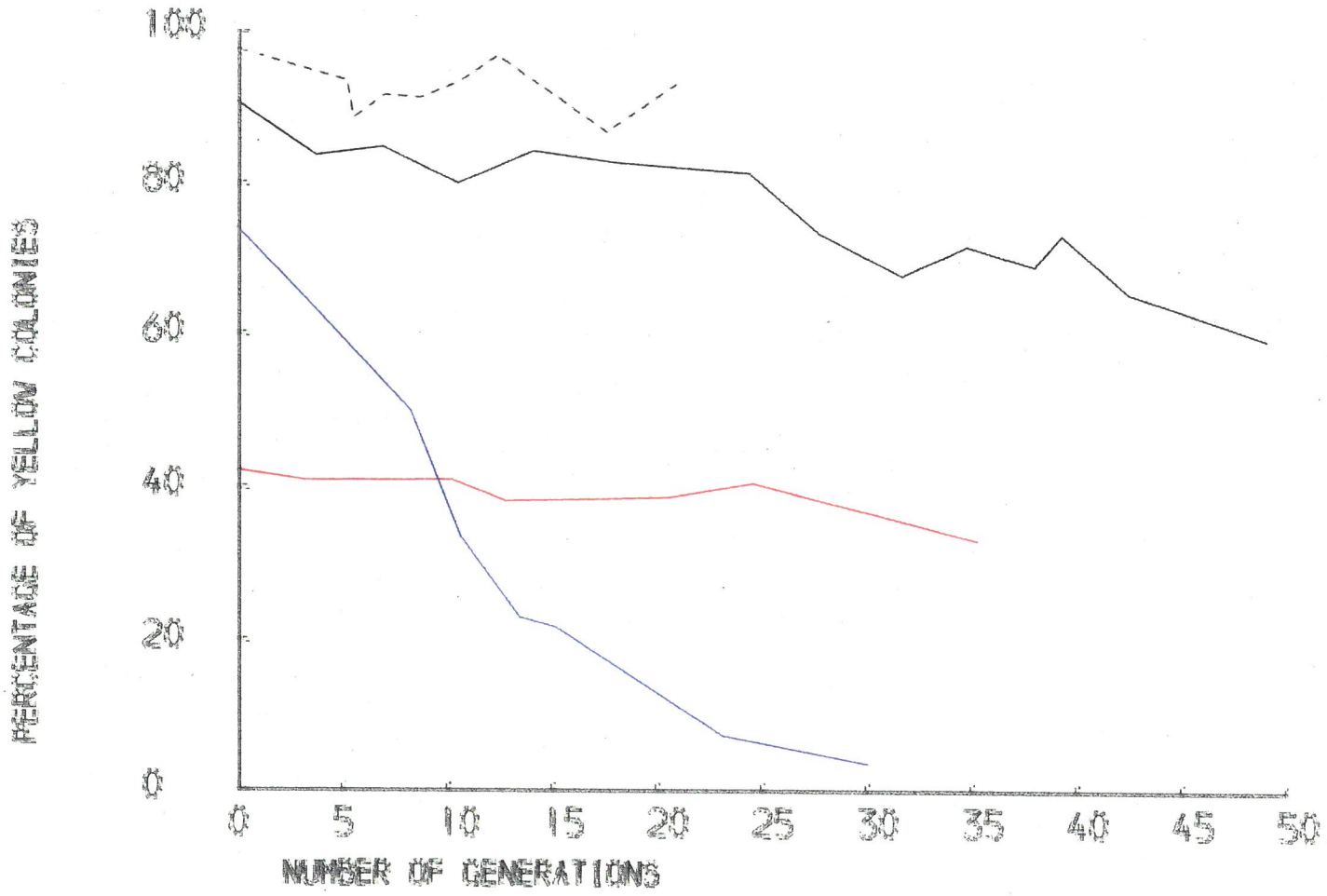


FIGURE 15 CHANGE IN PERCENTAGE OF YELLOW WILD TYPE IN POPULATIONS OF ARTHROBACTER GLOBIFORMIS GROWN IN CONTINUOUS CULTURE UNDER GLUCOSE LIMITATION. BLACK DASHED: D = 0.05 PER H, IN DARK. BLACK: D = 0.1 PER H, IN LIGHT. RED: D = 0.3 PER H, IN LIGHT. BLUE: D = 0.01 PER H, IN LIGHT.

CHAPTER 7. RELEVANCE OF THIS STUDY TO NATURAL ENVIRONMENTS

The aim of this study was to investigate the endogenous metabolism of a typical Arthrobacter species using several approaches and to assess its importance in the survival of this organism. The implications of the findings, particularly to the natural environment, will now be discussed.

Considerations of energy flow in natural soil eco-systems following input from leaf litter, root exudates, etc., to its termination in the biomass of the soil microflora, have indicated that bacteria are only able to grow very slowly. Babiuk and Paul (1970) calculated a growth rate of about $0.00058h^{-1}$ (generation time, g = 1200h) for bacteria in a grassland system but admit that this is an under-estimate since it was based on total counts. Calculations based on viable counts and carbon dioxide evolution from an agricultural soil gave a growth rate of $0.024h^{-1}$ (g = 28.5h) and estimates of growth rates in other natural habitats lie between $0.1h^{-1}$ (g = 7h) and $0.002h^{-1}$ (g = 300h) (Gray and Williams, 1971). The growth rate of the viable bacterial biomass in a deciduous woodland was calculated to be $0.0071\ h^{-1}$ (g = 100h) assuming a specific maintenance rate of $0.001h^{-1}$, a yield factor of 0.35 and ignoring fungal or animal growth (Gray, Hissett and Duxbury, 1974). If the bacteria use only 25% of the input organic matter but taking the yield factor to be 0.50, the mean growth rate becomes $\quad h^{-1}$ (g = h) (Hissett and Gray, 1976).

The assumed value of the yield factor has a large effect on the outcome of these calculations and a value of 0.60, as obtained for Arthrobacter globiformis NCIB 10683, is probably more realistic (Payne, 1970). The value of the specific maintenance rate, providing it is small, has a small effect when the energy input is large compared to the standing bacterial biomass. If a large fraction of the energy input is diverted to fungi, then the value of the specific maintenance rate becomes much more significant. The value of $0.001h^{-1}$ for the specific maintenance rate is lower than that obtained for A. globiformis NCIB 10683 at $0.002h^{-1}$ (μ_e at 10°C, see table 4). Shields, Paul, Lowe and Parkinson (1973) also estimated a specific maintenance rate of $0.002h^{-1}$ for the mixed fungal and bacterial populations of a brown chernozemic soil. Recalculation of the data of Gray et al. (1974) using the values obtained for A. globiformis, a revised formula (see Appendix V) and again assuming the bacteria use only 25% of the input organic matter, gives a growth rate of $0.0032h^{-1}$ ($g = 217h$).

All these calculated values for the growth rate of bacteria in soil are much lower than those obtained in normal batch culture but cells grown in continuous culture at dilution rates below $0.05h^{-1}$ may have a composition similar to those in nature. This assumes that the energy flow in the natural environment is constant but if it is not, due to environmental factors such as the rate of organic matter addition, temperature, water availability and pH, then the growth rate will fluctuate

and may reach higher values at least for short periods. Hissett and Gray (1976) found the growth rate of bacteria on Fraxinus leaflets, newly added to soil, to be $0.032h^{-1}$ ($g = 21.6h$) and a large proportion of the isolates from the ash litter in this woodland soil were found to be Arthrobacter species (Hissett and Gray, 1974). Duxbury (1973) suggested that it is unlikely that rod cells will be found in soil although rod to coccus morphogenesis occurs at a growth rate of $0.025h^{-1}$ ($g = 28h$) at $10^{o}C$ (Luscombe, 1972) and presumably at lower growth rates at lower soil temperatures. Hence rod cells could be found, particularly in the region of freshly-added substrate and at low temperatures. It is useful to consider in what way the composition of slowly-grown cells differs from that of rapidly-grown cells. In doing this it is important to bear in mind that soil temperatures are usually much lower than the $25^{o}C$ used in this study and that temperature can have an effect on cell composition independent of its effect on growth rate (Tempest and Hunter, 1965; Hunter and Rose, 1972).

The presence of "extra" RNA in carbon-limited A. globiformis will be advantageous in a fluctuating environment where it could be important in reducing the lag period before the organism could multiply following in increase in nutrient supply. Its presence in nitrogen-limited cells is also indicated but requires verification.

Both carbon and nitrogen-limited cells at low growth rates

appeared to have an increased cell density. At present it is unclear what mechanism or cell component is involved in this but it may be of importance in resistance to stress, particularly desiccation. It also means that each cell or viable unit has an increased mass upon which to depend in times of nutrient starvation.

Although Duxbury (1973) showed that the cell wall had no special structure, the high wall content in itself may aid resistance to lysis and desiccation.

Another important consideration is the nature of the limitation in the natural environment. Calculations of energy flow in soil suggest that the energy input, principally in the form of organic carbon, is hardly sufficient to support the mass of microorganisms present and certainly cannot support them growing at their maximum growth rate. This means that bacteria in soil are probably carbon-limited. However many soil environments have a high C/N ratio; the A_1 horizon from which A. globiformis NCIB 10683 was isolated has an overall C/N ratio of 32:1 (Goodfellow et al. 1968). The carbon-limited medium used in this study has a C/N ratio of 0.76:1 and the nitrogen-limited medium a C/N ratio of 26:1 indicating that the A_1 horizon could have a nitrogen-limited microflora. This is modified by the fact that the measured overall C/N ratio is not necessarily the same as that of the nutrients flowing through the system. A large proportion of the nitrogen in the soil is probably bound up in the microflora itself, thus the C/N ratio of the available nutrients is probably much higher than 32:1. The C/N ratios of the soil horizons above

the A_1 are progressively higher, the uppermost L horizon having a C/N ratio of 74:1 (Hill, 1967).

A second modification has the reverse effect. If the growth rate in soil is very low a large proportion of the available energy will be used in maintenance such that the microflora is no longer nitrogen-limited. This may be illustrated using A. globiformis.

Consider the production of 1g cells with a composition similar to that of carbon-limited cocci at a dilution rate of $0.01h^{-1}$. 1g cells contains 414.5 mg carbon and about 102.5mg nitrogen (assuming protein and nucleic acids are 16% nitrogen). With a yield factor of 0.60 this will require 690 mg carbon apart from that used in maintenance. In a nutrient supply with a C/N ratio of 32:1, for every 102.5 mg nitrogen there is 3280 mg carbon. Therefore 3280-690 = 2590 mg carbon will be available for maintenance. Taking a specific maintenance rate of $0.002h^{-1}$ the maintenance coefficient is 1.33 mg carbon $(g\ cells)^{-1}\ h^{-1}$. Therefore 2590 mg carbon will last 1g cells 1942h. With a mean generation time of 1942h ($\mu = 0.00036h^{-1}$) the cells will just use up all the available carbon source and none will be available for accumulation into polysaccharide: the cells are carbon-limited.

A similar calculation based on the composition of nitrogen-limited cocci at a dilution rate of $0.01h^{-1}$, i.e. cystites containing an appreciable quantity of intracellular polysaccharide, gives a mean generation time of 460h ($\mu = 0.0015h^{-1}$)

Since it is unlikely that A. globiformis can have a lower nitrogen content than these cystites grown in the chemostat, at growth rates above $0.0015h^{-1}$ carbon will remain unused and the cells will be nitrogen-limited. Again, if the energy flow in soil fluctuates, there could be times of carbon-limitation alternating with times of nitrogen-limitation and polysaccharide accumulation.

The low value of the specific maintenance rate obtained in the chemostat studies under growing conditions would argue for A. globiformis living in a carbon-limited environment although the experiments also indicate that "uncoupling" under nitrogen-limitation, particularly at low growth rates, is quite small. This would be important if the energy supply is fluctuating and at times limiting : wastage of energy would be deleterious. It appears that this organism can efficiently channel the excess carbon (energy) source into reserve polysaccharide, a feature typical of soil arthrobacters (Mulder and Zevenhuizen, 1967).

Although no experimental evidence was put forward, the implications of maintenance of non-viable cells, i.e. both senescent and moribund cells, is ecologically interesting. If in a population where cells that have become non-viable no longer require maintenance, energy is conserved and more is available to the viable populating giving it an advantage over populations where non-viable cells are also consuming energy. The application to human populations is less appealing.

The picture is modified under starvation conditions. The rate of endogenous metabolism is much lower during starvation than during growth: between 10 and 100-fold lower under carbon starvation and about 10-fold lower under nitrogen starvation. The higher rate in the nitrogen starved cells shows that the available energy source in the form of the endogenous reserve material, the intracellular polysaccharide, was not being used efficiently and this factor probably accounts for the observed increase in loss of viability during nitrogen starvation as compared to carbon starvation. It is possible that the polysaccharide energy reserve may be more important under stress conditions other than starvation which require a high energy input to counteract them such as ionic imbalance or low pH (Luscombe, 1972). It is concluded that carbon-limited cells, even if they have been growing slowly, will exhibit the best survival under straight-forward starvation in the soil environment.

Just as the maintenance requirement under growing conditions has been shown to decrease with temperature, so it is reasonable to assume that the rate of endogenous metabolism under non-growing conditions will decrease. Thus viability will be maintained much longer at soil temperatures than at 25°C although further experimentation is required to verify this point.

The mechanism by which A. globiformis can tolerate such a low level of endogenous metabolism also requires further investigation. It is probable that a low level of ATP such as

found in starved A. globiformis ATCC 15481 (Leps and Ensign, 1972) plays a part in this.

The studies on the white and yellow varieties of A. globiformis suggest that the white mutant would slowly replace the yellow wild type during the slow growth rates experienced in the soil. However Lowe (1969) found 7-10% of the bacterial population of the A_1 horizon to be yellow pleomorphic rods suggesting that the yellow pigment does confer some advantage. It is difficult to believe that this is photo-protection against the very low levels of light which must be experienced in the A_1 horizon and this effect may be purely fortuitous although of possible value to pigmented organisms in the upper litter layers.

Koch (1971) postulated that every microbial cell has a predicament, namely whether to prepare itself for feast or famine. The cell biochemistry and physiology needs to be suited to one or other of these states. It appears that Arthrobacter globiformis, representative of the soil autochthonous flora, has adapted itself to a famine existence.

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Appendix I. Properties of *Arthrobacter globiformis* NCIB 10683

These results were from the work of Lowe (1969) now stored as computer output in the Hartley Botanical Laboratories, University of Liverpool. Unapplicable or ambiguous results in the data have been omitted.

Cell length	0.8 - 1.0 µm
Cell width	up to 0.7 µm
Pleomorphic	
Reaction to Grain's stain, 24h	variable
Production of endospores	-
Motility	-
Colony elevation	convex
Colony shape	circular
Colony pigmentation	yellow, 7.5 - 7.8*
Diffusible purple pigment	-**
Production of fluorescent pigments	-
Surface growth in nutrient broth	pellicle or ring
Limits of growth on P.Y.E. agar at differing initial pH	no growth below 5.0, growth at 9.0
Limits of growth on nutrient agar containing different concentrations NaCl	growth at 7%, not above
Limits of growth on P.Y.E. agar at differing temperatures	growth at 35°C, not above, growth at 10°C

Utilization of sole nitrogen sources :	ammonium -**
	aspartate -
	cysteine -
	glutamate -
Utilization of sole carbon sources :	gluconate +
	lactate -
	tartrate +
	succinate +
	oxalate -
	p-hydroxybenzoate +
	alginate +
	citrate +
	acetate -
	formate -
	glucosamine -
	coumarin -
	vanillin -
	vanillic acid -
Growth in the presence of chemical agents :	phenol 0.1% w/v
	crystal violet 0.001% w/v
	sodium azide 0.01% w/v
	potassium tellurite 0.05% w/v
Acid production in open Hugh and Leifson tubes plus glucose	no acid
Acid production in closed Hugh and Leifson tubes plus glucose	no acid
Acid production in Hugh and Leifson medium.	No acid from D-arabinose, fructose, galactose, rhamnose, mannose, cellobiose, lactose, maltose, sucrose, trehalose, melizitose, raffinose, glycogen, inulin, amygdalin, salicin, glycerol, mannitol, inositol
Catalase production	+
Oxidase production	-
Indole production	-
Hydrogen sulphide production	produced after 7-14d
Urease activity	-
Acetyl methyl carbinol production	not produced after 14d
Nitrate reduction	+

Hydrolysis of :	gelatin	+
	aesculin	+
	xylan	+
	laminarin	+
	tributyrin	+
	cellulose	-
	chitin	-
Pectinase production		-
Decarboxylation of :	arginine	-
	lysine	-
	ornithine	-
Cystite production		+
Hydrolysis of casein		+

Antibiotic sensitivity: Weakly sensitive (zone of inhibition up to 2mm) to polymyxin B (50 units per disc), sensitive (zone of inhibition 2-10mm) to aureomycin (5mcg), bacitracin (2 units), penicillin (2 units), erythromycin (2mcg), novobiocin (5mcg), streptomycin (2mcg), chloromycetin (5mcg).

Reaction to Grain's stain, 48h	+
Hydrolysis of starch	-
Utilization of araban	-
Decolourization of humic acid	-
Degradation of lignin	-

* Rembrant colour chart, Talenz, Holland

** Subsequent studies indicate that these should be positive.

Appendix II. Definition of symbols used in theory of maintenance energy.

Symbol	Definition	Unit
D	dilution rate, unit volume of culture replaced per unit volume of culture per unit time	h^{-1}
D_2	effective evaporation rate	h^{-1}
F	flow rate of medium into culture	$ml\ h^{-1}$
F_2	evaporation rate of water into air stream	$ml\ h^{-1}$
f	fraction of substrate consumed by non-viable biomass as compared with consumption by viable biomass	
g	"mean" generation time	h
K_s	saturation constant	$g\ l^{-1}$
q^{nv}	rate of consumption of limiting substrate by non-viable biomass, unit mass of substrate consumed per unit non-viable biomass per unit time	$g\ g^{-1}\ h^{-1}$
q^{nv}_{max}	maximum theoretical value of q^{nv}	$g\ g^{-1}\ h^{-1}$
q^{nv}_e	rate of consumption of limiting substrate by non-viable biomass for use in endogenous metabolism	$g\ g^{-1}\ h^{-1}$
q^{v}	rate of consumption of limiting substrate by viable biomass, unit mass of substrate consumed per unit viable biomass per unit time	$g\ g^{-1}\ h^{-1}$
q^{v}_{max}	maximum theoretical value of q^{v}	$g\ g^{-1}\ h^{-1}$
q^{v}_e	rate of consumption of limiting substrate by viable biomass for use in endogenous metabolism	$g\ g^{-1}\ h^{-1}$
s	concentration of limiting substrate in the culture	$g\ l^{-1}$
S_r	concentration of limiting substrate in the inflowing medium	$g\ l^{-1}$
t	time	h
V	viability of culture, number of viable cells as a fraction of the total number of cells	
v	working volume of culture	ml
x^{nv}	concentration of non-viable biomass	$g\ l^{-1}$
x^{T}	concentration of total biomass	$g\ l^{-1}$
x^{v}	concentration of viable biomass	$g\ l^{-1}$
Y	observed yield factor, unit biomass formed per unit substrate added to culture	$g\ g^{-1}$
Y_{max}	maximum theoretical value of yield factor	$g\ g^{-1}$
α	index of viability, the probability that a newly-formed cell is viable	
μ	specific growth rate of population, units total biomass formed per unit viable biomass per unit time	h^{-1}
μ_{max}	maximum theoretical value of μ	h^{-1}
μ_{min}	minimum theoretical value of μ	h^{-1}
μ_d	specific death rate of population, units non-viable biomass formed per unit viable biomass per unit time	h^{-1}
μ_e	specific maintenance rate of viable biomass	h^{-1}
μ^{nv}	specific growth rate of non-viable biomass, units non-viable biomass formed per unit non-viable biomass per unit time	h^{-1}
μ^{nv}_e	specific maintenance rate of non-viable biomass	h^{-1}
μ^*	specific growth rate of population defined in terms of the "mean" generation time	h^{-1}

Appendix III. Calculation of Y_{glu}; two methods.

Y_{glu} is the yield of cells in grams per mole of glucose used as energy source.

Method 1. All non-cellular carbon oxidized

Yield = Y_{max} g cells / g glucose

= Y_{max} X 0.4145 g carbon / g glucose

(Carbon in glucose-limited cells = 41.45%, table 13)

= Y_{max} X 0.4145 / 0.4000 g glucose incorporated / g glucose used

(Carbon in glucose = 40.0%)

Therefore, glucose utilized as energy source to give Y_{max} g cells

= 1 - Y_{max} X 0.4145 / 0.4000 g

Therefore, 1 g gives (Y_{max} X 0.400) / (0.400 - Y_{max} X 0.4145) g cells

Hence, 1 mole gives $\frac{180.2 \times Y_{max} \times 0.400}{0.400 - Y_{max} \times 0.4145}$ g cells = Y_{glu}

For Y_{max} = 0.651 g g^{-1}

Y_{glu} = 360.5 g $mole^{-1}$.

Method 2. Material balance

Composition of carbon-limited cells (using mean values from table 13 and assuming the N:O ratio is the same as in Torulopsis utilis (Chen, 1964)):

C 41.45%, H 6.47%, N 7.82%, O 34.30%, Ash 9.96%.

Consider the material balance for the production of Y_{max} g cells,

z g glucose + w g NH_3 + 0.0996 X Y_{max} g ash $\longrightarrow$

Y_{max} g cells + x g CO_2 + y g H_2O

From this three equations can be obtained by considering the balances of the elements hydrogen, carbon, oxygen and nitrogen:

H: $\frac{12}{180}z + \frac{3}{17}w = Y_{max} \times 0.0647 + \frac{2}{18}y$

C: $\frac{72}{180}z = Y_{max} \times 0.4145 + \frac{12}{44}x$

$$O: \frac{96}{180}z = Y_{max} \times 0.3430 + \frac{32}{44}x + \frac{16}{18}y$$

$$N: \frac{14}{17}w = Y_{max} \times 0.0782$$

Solving these simultaneous equations obtains z. Since 1 g glucose has been used to give Y_{max} g cells, (1 - z) g glucose has been completely oxidized.

Therefore, 1 g glucose gives $Y_{max} / (1 - z)$ g cells

1 mole glucose gives $180.2 \times Y_{max} / (1 - z)$ g cells = Y_{glu}

For $Y_{max} = 0.651$ g g^{-1}

$z = 0.699$ g

$Y_{glu} = 389.5$ g $mole^{-1}$.

Appendix IV. Conversion of oxygen uptake ($Q[O_2]$) values into their equivalent maintenance coefficients.

Consider an oxygen uptake rate,

$$Q[O_2] = 1\ \mu l\ [O_2]\ \text{(mg cells)}^{-1}\ h^{-1}$$

$$= 10^{-6}/22.4 \text{ moles } [O_2] \text{ (mg cells)}^{-1}\ h^{-1}$$

(1 mole of oxygen at N.T.P. occupies 22.4 l)

$$= 10^{-3}/22.4 \text{ moles } [O_2] \text{ (g cells)}^{-1}\ h^{-1}$$

Assuming the oxygen is used in the complete oxidation of substrate in a form equivalent to glucose :

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O\ ,$$

this quantity will oxidise

$10^{-3}/(22.4 \times 6)$ moles glucose $(\text{g cells})^{-1}\ h^{-1}$

$= 180 \times 10^{-3}/(22.4 \times 6)$ g glucose $(\text{g cells})^{-1}\ h^{-1}$

i.e. a maintenance coefficient

$= 0.00134$ g glucose $(\text{g cells})^{-1}\ h^{-1}$

Taking $Y_{max} = 0.602$ g cells $(\text{g glucose})^{-1}$ this gives an equivalent specific maintenance rate

$$= 0.000806\ h^{-1}.$$

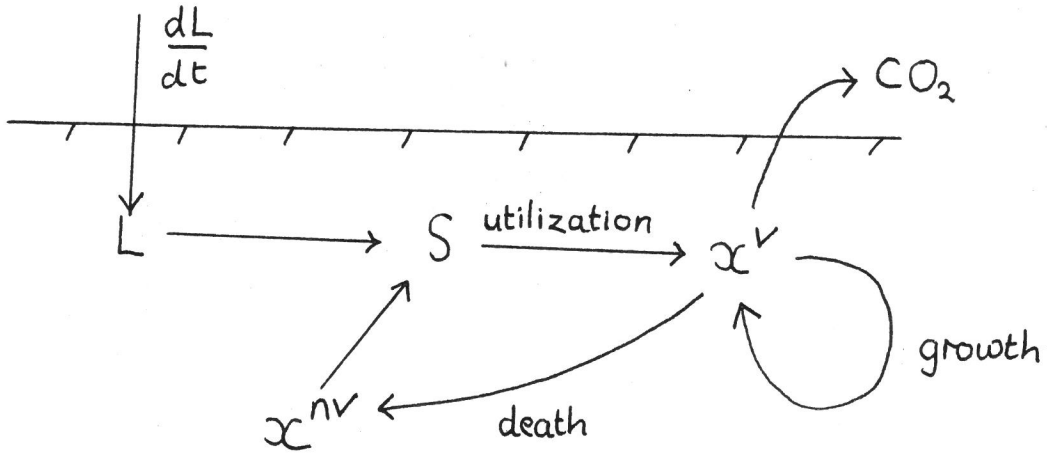
Appendix V. Revised formula for the growth rate of bacteria in soil based on energy input.

This is considered to be more exact than the formula given by Gray and Williams (1971): $Y(S + xR) = xR$, where R is the number of generations per year.

Assumptions:

(i) that dead cells are utilized as substrate with equal efficiency to that of litter.

(ii) that bacteria do not utilize dead fungi and vice versa.



Symbols other than given in appendix II:

S	total substrate in soil	g
L	total litter in soil	g
μ_c	specific consumption rate of non-viable cells	h^{-1}

Taking one hectare of the soil system, consider the change in total bacterial biomass:

$$dx^T/dt = \underset{\text{(growth)}}{\mu x^v} - \underset{\text{(consumption of dead cells)}}{\mu_c x^{nv}}$$

Consider change in non-viable biomass:

$$dx^{nv}/dt = \underset{\text{(death)}}{\mu_d x^v} - \underset{\text{(consumption)}}{\mu_c x^{nv}}$$

At steady state:

$$dx^T/dt = 0 \qquad dx^{nv}/dt = 0$$

Therefore:

$$\mu x^v = \mu_c x^{nv} = \mu_d x^v \qquad \ldots\ldots\ldots\ldots (1)$$

Hence:

$$\underset{(\text{growth})}{\mu} = \underset{(\text{death})}{\mu_d}$$

Consider change in substrate:

$$dS/dt = \underset{(\text{litter input})}{dL/dt} + \underset{(\text{dead cells input})}{\mu_c x^{nv}} - \underset{(\text{consumption by viable cells})}{q^v x^v}$$

At steady state:

$$dS/dt = 0$$

Therefore:

$$q^v x^v = dL/dt + \mu_c x^{nv}$$

From (1):

$$q^v x^v = dL/dt + \mu x^v$$

$$\underset{(\text{growth})}{x^v \mu / Y_{max}} + \underset{(\text{maintenance})}{x^v \mu_e / Y_{max}} = dL/dt + \mu x^v$$

$$\mu x^v + \mu_e x^v = Y_{max} dL/dt + \mu x^v$$

$$\mu x^v (1 - Y_{max}) = Y_{max} dL/dt - \mu_e x^v$$

$$\mu = \left[\frac{Y_{max}}{x^v} \cdot \frac{dL}{dt} - \mu_e \right] / (1 - Y_{max})$$

Taking,

$$Y_{max} = 0.60$$

$$\mu_e = 0.002 \ h^{-1}$$

$$x^v = 36870 \ g$$

$$dL/dt = 201.5 \ g \ h^{-1} \ (25\% \text{ Of total litter input. })$$

$$\mu = 0.0032 \ h^{-1}$$

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"Of making many books there is no end, and much study is a weariness of the flesh."

Ecclesiastes 12:12